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An investigation into the biochemistry of β2glycoprotein I and the interaction of the fibrinolytic system with antiphospholipid antibodies

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Abstract

The antiphospholipid syndrome (APS), is an autoimmune condition characterised by the occurrence of thrombosis (both arterial and venous) and obstetric morbidity along with the persistent production of antiphospholipid antibodies (aPL).

At present the pathophysiology of APS is unclear although several hypothesis are available in the literature. This thesis has aimed to examine some of the processes behind the APS focusing on the interaction of aPL with components of the fibrinolytic system and the interaction of Beta2 glycoprotein I (β2GPI) with fibrinolytic and other proteolytic enzymes.

A detailed examination of our patient cohort was undertaken and compared to new serological criteria for APS. The interaction of aPL on plasmin mediated cleavage of β2GPI was examined and found to be reduced in the presence of some aPL. Plasma kallikrein and Xa were tested for proteolytic activity on β2GPI and found to posses this although to a lesser degree to that seen for plasmin. The effect of domain V genetic polymorphisms of β2GPI on the action of plasmin on this part of the molecule was examined and found to be reduced in the presence of these polymorphisms. An investigation into the effect of one of these polymorphisms (Cys306Gly) on aPL production was undertaken with negative results in this respect. An examination of the effect of aPL on the binding of plasminogen to endothelial cell surfaces was undertaken and in some patient samples found to have an effect on this process. Moreover, some aPL were found to reduce fibrinolysis in a plasma clot lysis assay.

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In particular I thank Ray, Andrew, Gordon and Richard for their "beyond the call of duty" help with many of the practical aspects of this work and willingness to discuss issues relating to my work and answer my many questions.

Abbreviations used in this thesis

APS	Antiphospholipid syndrome
ABS	Adult Bovine Serum
aCL	Anticardiolipin antibodies
ADH	Antidiuretic Hormone
α2PI	Alpha 2 plasmin inhibitor
APC	Activated protein C
APCR	Activated protein C resistance
aPL	Antiphospholipid antibodies
APOH	Apolipoprotein H
APTT	Activated partial thromboplastin time
AT	Antithrombin
β2GPI	Beta 2 glycoprotein I
BSA	Bovine serum albumin
CAM	Cell adhesion molecule
CVA	Cerebro Vascular Accident (Ischaemic stroke)
DRVVT	Dilute Russell's Viper Venom time
EACA	ε-amino caproic acid
EC	Endothelial Cell
EPCR	Endothelial protein C receptor
FITC-Pg	FITC-Plasminogen
HDL	High Density Lipoprotein
HMWK	High Molecular Weight Kininogen
HRP	Horse Radish Peroxidase
HUVEC	Human Umbilical Vein Endothelial Cells
kDa	kilo Daltons
KIU	Kallikrein inhibition units
LA	Lupus anticoagulant
LDL	Low density lipoprotein
PAR	Protease activated receptor
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction

PFA	Paraformaldehyde
PNP	Pooled normal plasma
PRCP	Prolylcarboxypeptidase
PT	Prothrombin time
PZ	Protein Z
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SLE	Systemic lupus erythematosis
TAFI	Thrombin activated fibrinolysis inhibitor
TF	Tissue factor
TFPI	Tissue factor pathway inhibitor
TMB	Tetramethylbenzidine dihydrochloride
TNF	Tumour necrosis factor
tPA	Tissue plasminogen activator
TVT	Taipan venom time
uPA	Urokinase
UPAR	Urokinase receptor
VTE	Venous thromboembolic event
VWF	von Willebrand factor

Chapter 1 Introduction

1.1 Normal Haemostasis

The coagulation mechanism requires an interaction between components of plasma, platelets, the vascular endothelium and subendothelium. In this section the physiology of clot formation will be discussed with particular emphasis on the "cell based" model of coagulation and the haemostatic function of the vascular endothelium. A discussion of the fibrinolytic system will follow.

A detailed description of the physiology of platelets is not presented here. It is however vital to remember that platelet function is closely linked to the coagulation mechanism and vital for normal haemostasis. Platelets, via their interaction with the subendothelium (via collagen, von Willebrand factor and the platelet Ib/IX/V receptor), subsequent activation and aggregation (via fibrinogen/fibrin binding the platelet IIb/IIIa receptor), provide the first wave of primary haemostasis. Moreover, activated platelets, via membrane "flip-flop", provide a negatively charged phospholipid surface for the assembly of the tenase and prothrombinase complexes of the coagulation mechanism. Activation of the clotting mechanism serves to reinforce platelet activation via the platelet agonist actions of thrombin mediated by the PAR_1 and PAR_4 receptors. A recent review of platelet physiology is available in the literature (Watson SP & Harrison P, 2005).

1.1.1 The coagulation mechanism

In the mid 1960's, two groups proposed a "waterfall" like mechanism for the coagulation mechanism with each clotting factor being converted into its active form by the preceding factor in the cascade (Macfarlane, 1964), (Davie & Ratnoff, 1964) (Figure 1). The components of these two systems are measured clinically in the Prothrombin time (extrinsic system) and activated partial thromboplastin time (intrinsic system). This traditional view of coagulation with two parallel systems an "intrinsic" and "extrinsic" system has recently being revised.

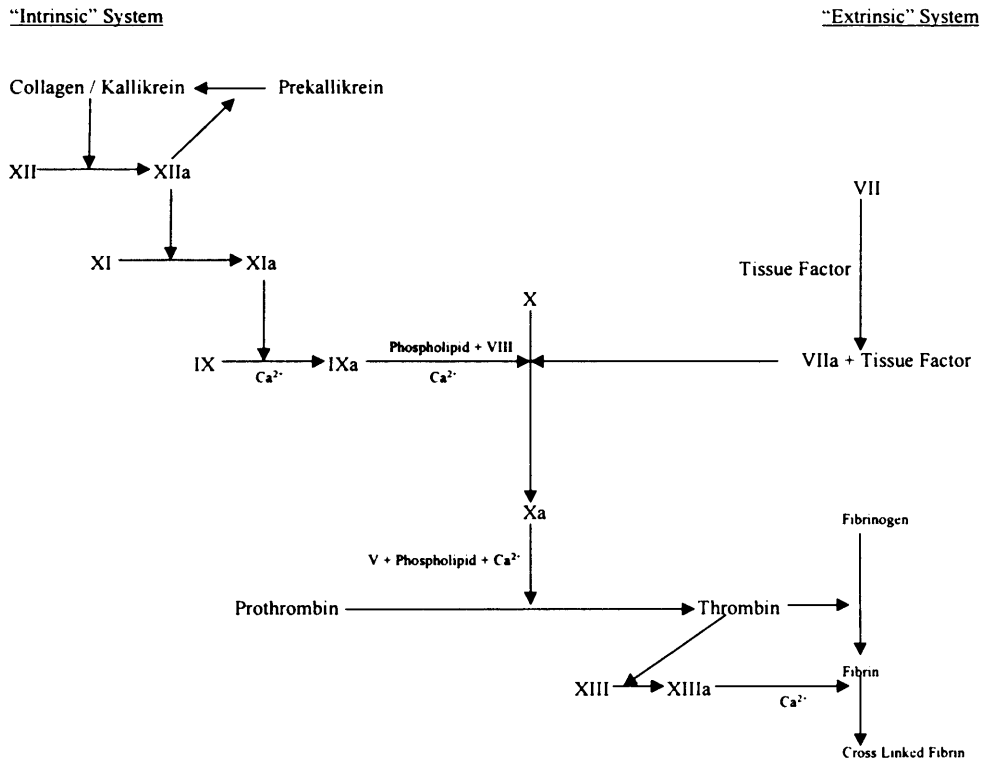


Figure 1 The cascade mechanism of blood coagulation.
Adapted from Davie and Ratnoff (1964)

This model of coagulation cannot however explain some clinical observations such as the absence of a bleeding tendency in patients deficient for factor XII or kallikrein and lack of compensation for deficiency of factors VIII or IX by the extrinsic system. A revised model for the coagulation mechanism based on a close interplay between the coagulation factors and cellular components (notably platelets and endothelial cells) has been proposed. This hypothesis has been recently reviewed (Hoffman, 2003a); (Hoffman, 2003b); (Hoffman & Monroe, III, 2001).

By localising the coagulation reactions to cellular surfaces, it is possible to achieve a powerful procoagulant burst at the site of injury while ensuring that the process remains localised. This cell-based model of coagulation can be described in terms of initiation, amplification and propagation and requires the presence of tissue factor (TF) bearing cells and platelets.

The initiation step in coagulation is the activation of factor VII in a TF.VII complex at the surface of a TF expressing cell (e.g. an activated monocyte or a cell at a site of damaged vascular endothelium). TF is an integral membrane protein (and thus remains localised to the cell it was synthesised by). It is expressed under basal conditions by extravascular cells and by vascular endothelial cells and monocytes under inflammatory stimuli. A breech in the vessel wall allows TF and VII from plasma to come into contact resulting in the activation of factor VII. Factor Xa produced by the action of the TF.VIIa complex forms a prothrombinase complex (factor Xa can act on factor V to produce the factor Va needed for this step). This reaction is limited to the locale of the TF bearing cell by virtue of the inhibitory action of tissue factor pathway inhibitor (TFPI) and antithrombin (AT) in plasma. This initiation step is likely to

occur constantly (at a low level) under normal conditions, it can however only proceed to an amplification and propagation phase when platelets and factor VIII.von Willebrand factor (VWF) complexes spill out of the vascular space. The activity of the TF.VIIa complex is quickly inhibited by TFPI. The initiation process is summarised in Figure 2.

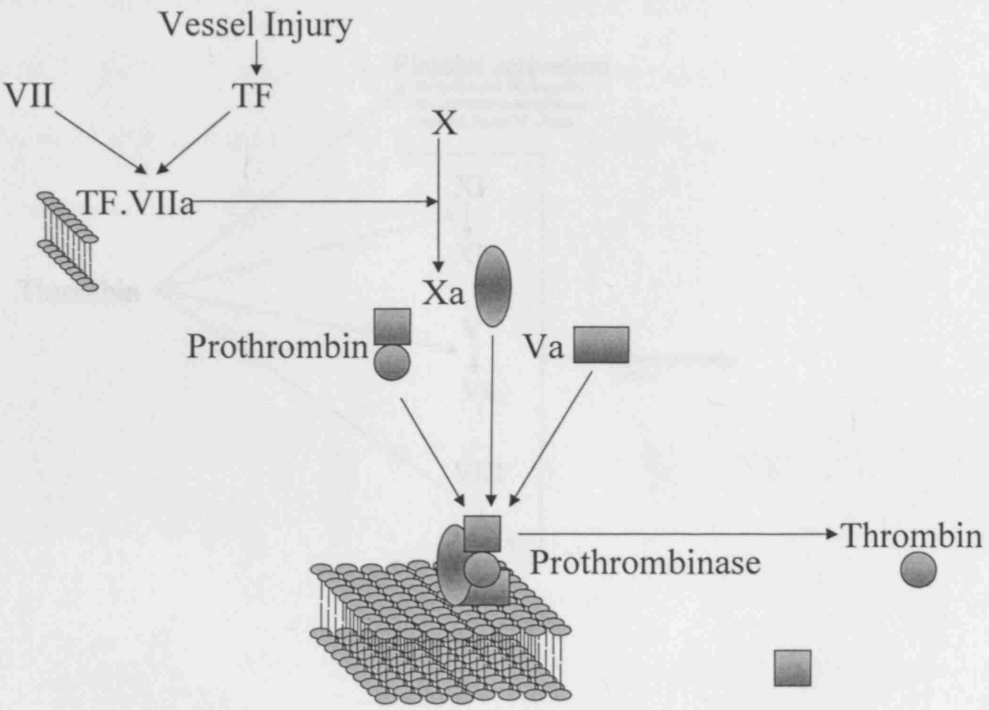


Figure 2 The initiation phase of coagulation.

Vessel injury results in the exposure of TF bearing cells. The TF.VIIa complex activates factor X, which in concert with factor Va and the cell surface forms a prothrombinase complex. TF.VIIa also activates factor IX.

Amplification of coagulation occurs at the surface of platelets activated partly by the thrombin produced in the initiation phase. Thrombin produced in the activation phase activates factors V, VIII and XI at the platelet surface (figure 3). The activating action of thrombin on platelets is transduced by protease-activated receptors (PAR) at the platelet surface.

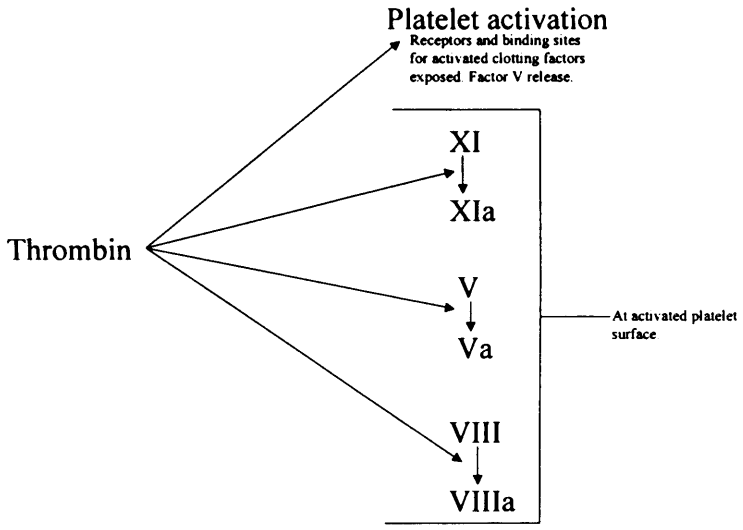


Figure 3 Amplification phase of coagulation

The propagation phase of coagulation takes place at the surface of activated platelets recruited to a site of vascular injury. The TF.VIIa complex formed in the initiation phase can activate factor IX as can factor XIa formed during the amplification phase by thrombin. Factor IXa combines with factor VIIIa to form a tenase complex at the platelet surface. Factor Xa thus formed combines with factor Va formed during the amplification phase to form prothrombinase; the resulting thrombin burst is sufficient to clot fibrinogen (figure 4). Factor V in partly activated form is also released from the alpha granules of activated platelets. The role of factor XI in this scheme is probably to enhance the amount of platelet bound factor IXa.

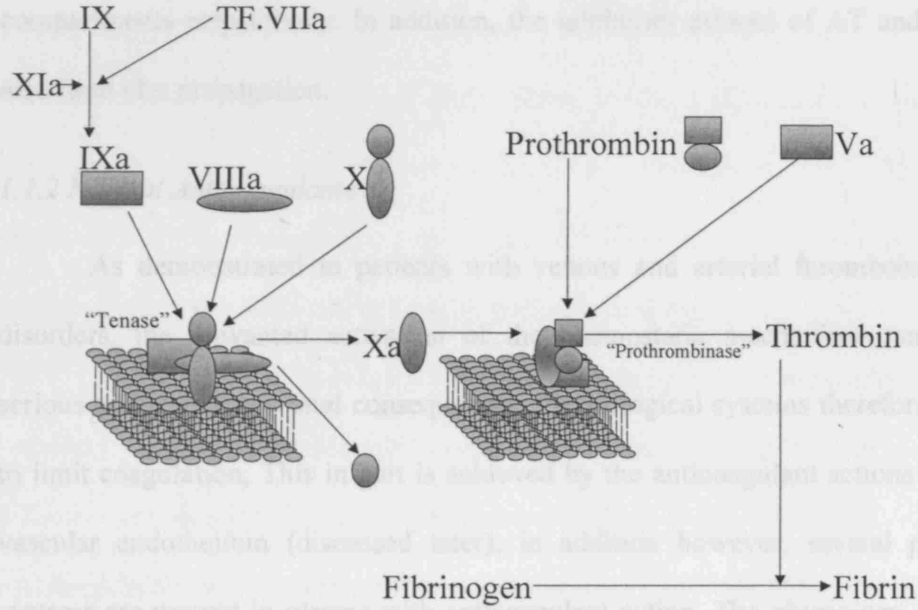


Figure 4 Propagation phase of coagulation

The propagation phase occurs at the surface of activated platelets.

Factor XIII, is a transglutaminase enzyme which covalently cross-links polymerised fibrin. The action of this enzyme is essential for a stable clot structure and deficiency of factor XIII is associated with a bleeding diathesis.

The uncontrolled propagation of this process is in part limited by thrombin itself. Thrombin binds thrombomodulin on intact vascular endothelial cells. The so formed thrombin-thrombomodulin complex activates protein C to activated protein C (APC). APC in complex with its cofactor protein S cleaves and inactivates factors Va and VIIIa. The coagulation system itself can also be seen to be self limiting in that it requires the close juxtaposition of TF bearing cells and activated platelets which are usually in the extravascular and vascular compartments respectively. In addition, the inhibitory actions of AT and TFPI also limit clot propagation.

1.1.2 Natural Anticoagulants

As demonstrated in patients with venous and arterial thromboembolic disorders, the unwanted activation of the haemostatic mechanism can have serious and sometimes fatal consequences. Physiological systems therefore exist to limit coagulation, This in part is achieved by the anticoagulant actions of the vascular endothelium (discussed later); in addition however, several protein systems are present in plasma with anticoagulant action. The physiology of this process has recently being reviewed (Kemball-Cook G, 2005).

Tissue factor pathway inhibitor (TFPI) is a kunitz type protease inhibitor found in association with glycosaminoglycans at the endothelial surface. The physiological role of TFPI is to limit activity of the tissue factor-VIIa-X complex

by forming a quarternary complex with factor VIIa/TF/factor X, thus inhibiting initiation of the clotting mechanism.

Circulating Serine protease inhibitors (Serpins) are also responsible for limitation of coagulation. Physiologically the most important of these is antithrombin (AT) which forms a 1:1 irreversible complex with thrombin and also factors IXa, XIa, Xa, and kallikrein. The liver rapidly clears thrombin-antithrombin complexes. The antithrombin and anti Xa activity of AT is greatly enhanced (>2,000 fold) by heparin. The serpin heparin cofactor II inhibits thrombin and has its activity increased approximately 1,000 fold in the presence of heparin (although the main physiological cofactor appears to be dermatan sulphate). Other serpins with activity on the clotting mechanism include α1-antitrypsin (XIa and Xa) and C1 esterase inhibitor (factor XIIa and kallikrein). The recently described protein Z (PZ) system involves PZ-dependent protease inhibitor (a serpin) and its cofactor the vitamin K dependent protein Z. The main target of the PZ system is Xa. α2-macroglobulin is a non-serpin, which binds and inhibits (by steric hindrance) kallikrein, thrombin and Xa.

The activity of the cofactors in the tenase and prothrombinase complexes – factors VIIIa and Va respectively are limited by the action of protein C and its cofactor protein S (both of these proteins are vitamin K dependent). Protein C is activated by the action of thrombin when in complex with thrombomodulin. The activation of protein C is also increased via the interaction of the protein with the endothelial protein C receptor (EPCR). APC interacts with protein S at the phospholipid surface of activated platelets. APC inactivates Va and VIIIa via enzymic cleavage in the A domain of the proteins. The activity of protein S can

be reduced by increased circulating levels of C4b-binding protein to which it binds.

1.1.3 The role of the vascular endothelium in normal haemostasis

The vascular endothelium is vital to normal haemostatic function. The structure and function of vascular endothelial cells has recently being reviewed (Cines *et al*, 1998); (Sporn LA & Huber P, 2001). The vascular endothelium lines the surface of all the blood vessels. These cells reach a mass of approximately 1Kg in an adult human with a surface area of 1-7m^2. As well as providing a physical barrier between components of the coagulation system and sub-endothelial activators such as collagen and tissue factor, the vascular endothelium has an active physiological role in the coordination of haemostasis.

In the resting state, the vascular endothelium exerts an anticoagulant effect. This is afforded by several mechanisms including the secretion and presence at the endothelial surface of heparan sulphate and the surface expression of thrombomodulin (Esmon & Owen, 1981) required for the activation of protein C as well as thrombin activated fibrinolysis inhibitor (TAFI). In addition, via the synthesis and secretion of prostacyclin and nitric oxide, the vascular endothelium exerts a vasodilatory and anti-platelet effect under basal conditions. Endothelial cells (EC) also express the EPCR. EC also modulate fibrinolysis via secretion of tissue plasminogen activator (tPA) and urokinase (uPA). Moreover, EC caveolae (spherical invaginations of the plasma membrane) express the urokinase receptor (UPAR). EC also express TFPI, which inhibits Xa activation in the TF.VIIa.X complex. The release of TFPI from EC is increased by heparin.

EC contain granules known as Weibel-Palade bodies. Some of the VWF synthesised by EC is stored in these granules. However, most of the VWF produced by EC is constitutively released (about 5-10% is stored) (Hannah *et al*, 2002).

A variety of stimuli can result in EC changing their phenotype from an anticoagulant to a procoagulant state. These include thrombin, hypoxia, sheer stress and inflammatory cytokines such as TNFα and Il-1. The synthesis of tissue factor and PAI-1 is increased by cytokine exposure as well as by endotoxin. Concurrently, activated EC reduce their surface expression of thrombomodulin. EC activation also increases the surface expression of integrins and intercellular adhesion molecules such as ICAM-1 and VCAM. ICAM-1 and VCAM bind leucocyte integrins. Activated EC also secrete chemokines such as monocyte chemoattractant protein and Il-8. Vasoconstriction is promoted by activated EC secreting platelet activating factor and endothelin-1. Two types of EC activation are described: type I involves the secretion of pre-stored cellular components, type II activation takes longer and occurs via changes in protein synthesis. Thrombin can cause both type I and type II activation.

The synthetic hormone desmopressin (an ADH analogue) can increase the release of VWF multimers from the Weibel- Palade bodies in which they are stored.

The role of vascular EC in the pathophysiology of the antiphospholipid syndrome and details of its role in the binding of components of the fibrinolytic system and the contact system is discussed in later sections.

1.2 The contact system

The contact system consists of factor XII, Prekallikrein and high molecular weight kininogen (HMWK). The activation of factor XII downstream in a cascade involving (pre) kallikrein and high and low molecular weight kininogen has long been known to be the initiating event in the activated partial thromboplastin time (APTT) assay. However recent advances in the understanding of coagulation physiology have resulted in a shift in our understanding of the role of these factors *in vivo*. As described in previous sections normal coagulation can function without the input of the contact system. Moreover a physiological equivalent of the negatively charged APTT reagent has not been found. In addition deficiencies of factor XII or prekallikrein are not associated with a bleeding diathesis (Colman, 2001).

1.2.1 Contact system activation in vivo

Rather than requiring a negatively charged surface for activation, the contact system *in vivo* is activated at endothelial cell surfaces by means of a protein receptor complex. The process of contact activation at endothelial surfaces is summarised in figure 5. A high molecular weight kininogen – prekallikrein complex binds to a complex of gC1qR/uPAR/CK1 at endothelial cell surfaces in the presence of Zn^{2+}. The enzyme prolylcarboxypeptidase (PRCP) at the cell surface catalyses the activation of prekallikrein. Kallikrein subsequently cleaves high molecular weight kininogen to release bradykinin and may also catalyse the activation of factor XII. Recombinant PRCP has recently been shown to activate prekallikrein *in vitro* (Shariat-Madar *et al*, 2004). The bradykinin released in this process can stimulate endothelial cell release of nitric

oxide, tPA and prostacyclin (PgI_2). Factor XII has been shown to interact with the CK1/uPAR/gC1qR complex shown in figure 5 (Mahdi *et al*, 2002). The binding of factor XII to this complex is more regulated than that of the HK.PK complex. Factor XII binding needs a relatively high zinc concentration which is probably only achieved in the presence of activated platelets.

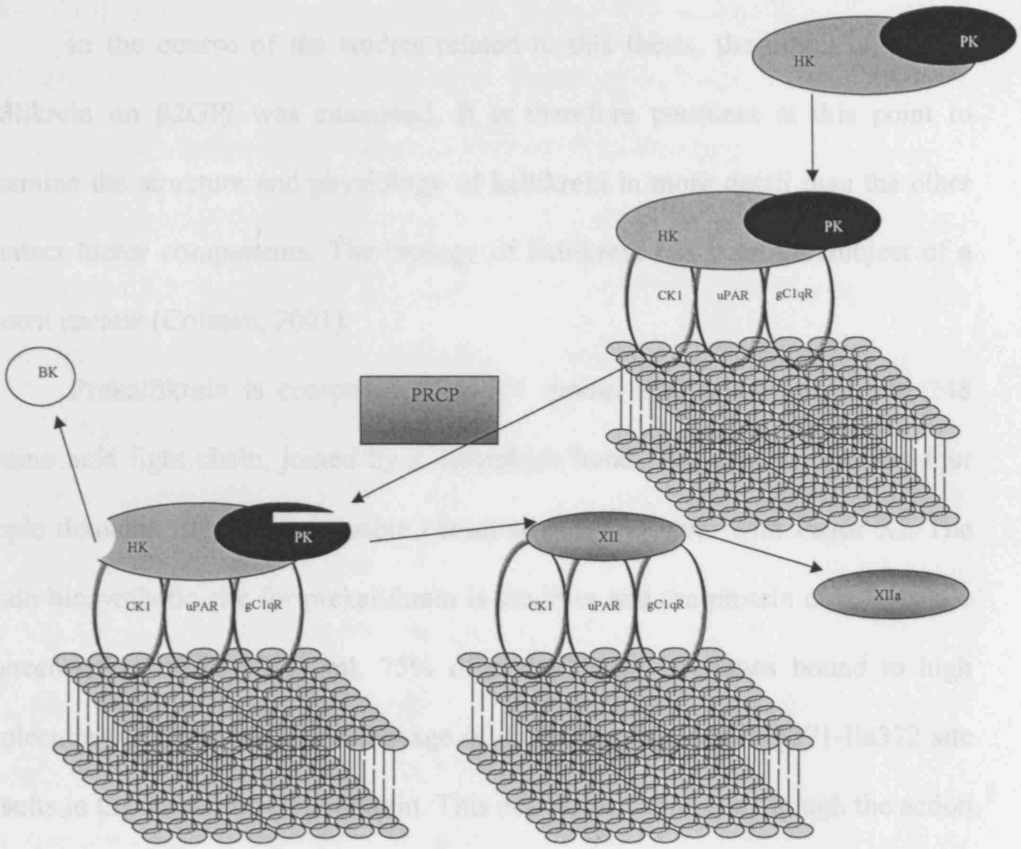


Figure 5 Assembly and activation of the contact system on endothelial cells

Adapted from Schmaier 2002 (Schmaier, 2002). Circulating plasma Prekallikrein (PK) in complex with high molecular weight kininogen (HK) binds to an endothelial cell membrane located complex of uPA receptor (uPAR), cytokeratin 1 (CK1) and the complement receptor gC1qR. The enzyme prolylcarboxypeptidase (PRCP, constitutively active at the endothelial surface) converts Prekallikrein to kallikrein. Kallikrein acts on HK to release bradykinin and activates factor XII, which also binds to the CK1/uPAR/gC1qR complex in the absence of HK.

An alternative mechanism for contact activation is probably important *in vitro* for example in the APTT assay. Here the binding of factor XII to a

negatively charged surface results in the autoactivation of factor XII to XIIa. Factor XIIa can then in the presence of HMWK, activate factor XI and prekallikrein. The kallikrein thus produced can then activate more factor XII in a positive feedback loop.

1.2.2 Plasma Kallikrein

In the course of the studies related to this thesis, the effect of plasma kallikrein on β2GPI was examined. It is therefore pertinent at this point to examine the structure and physiology of kallikrein in more detail than the other contact factor components. The biology of kallikrein has been the subject of a recent review (Colman, 2001).

Prekallikrein is composed of a 371 amino acid heavy chain and 248 amino acid light chain, joined by a disulphide bond. The protein contains four apple domains suggesting possible common ancestral gene with factor XI. The main biosynthetic site for prekallikrein is the liver and the protein circulates at a concentration of 35-50 μg/ml. 75% of prekallikrein circulates bound to high molecular weight kininogen. Cleavage of prekallikrein at the Arg371-Ile372 site results in the formation of kallikrein. This activation can occur through the action of factor XIIa and PRCP at the endothelial surface as described above. The active site of kallikrein (Ser559, His415 and Asp464) is contained in the light chain of the protein. The activity of kallikrein is inhibited *in vivo* by alpha2-macroglobulin, antithrombin and C1 esterase inhibitor, which interact with the light chain of the protein. (Deficiency of C1 esterase inhibitor results in the clinical manifestations of hereditary angioedema consequent on unchecked kallikrein activation and bradykinin release). The main physiological substrates

of kallikrein are factor XII, pro-urokinase and high molecular weight kininogen.

1.2.3 Physiological consequences of contact activation

Deficiency of one of the contact factors is a not-uncommon cause of the clinical finding of an isolated prolongation of the APTT in a patient. While such deficiencies are not associated with a bleeding diathesis, this should not undermine the importance of the contact factors in normal physiology.

Some of the physiological consequences of contact activation are as follows:

1) Bradykinin release. Bradykinin is one of the most potent vasodilators known. Bradykinin has potent biological effects including endothelial release of PgI_2 (which has anti-platelet and vasodilatory effects), nitric oxide and tissue plasminogen activator (tPA). These effects are mediated via G-protein coupled B1 and B2 receptors.
2) Inhibition of alpha-thrombin mediated activation of platelets. (Via HMWK inhibition of platelet calpain which is needed for platelet fibrinogen receptor activation) (Colman & Schmaier, 1997).
3) Activation of fibrinolysis. Kallikrein and XIIa can cleave and activate plasminogen directly but much less efficiently than tPA or uPA. Kallikrein can also activate uPA (from single chain to two chain form) and as mentioned before bradykinin is a stimulator of endothelial tPA release.

Thus in contrast to its apparent procoagulant role in the APTT assay, it can be seen that contact activation *in vivo* will have a net anticoagulant and pro-fibrinolytic effect.

1.3 The fibrinolytic system

1.3.1 Overview

The fibrinolytic system represents the physiological means to remove formed blood clots. More recently, the importance of this system in the process of tissue remodelling, cancer metastases and the invasion of the trophoblast into the endometrium has become apparent. Paramount to the optimum functioning of the fibrinolytic system is a tight balance between fibrinolytic activators, fibrinolytic enzymes and their inhibitors. This section will provide an overview of the fibrinolytic system. The physiology of fibrinolysis has been the subject of a recent review (Cesarman-Maus & Hajjar, 2005).

The major circulating plasma zymogen involved in fibrinolysis is plasminogen. This zymogen is converted to plasmin by the action of tissue plasminogen activator (tPA) and urokinase (uPA). Fibrin polymer enhances the activation of plasminogen by tPA by binding and co-localising the two proteins. (The activation of plasminogen by tPA is increased two-fold in the presence of fibrin). Moreover, plasmin itself provides positive feedback in this process by catalysing the conversion of single chain uPA and tPA to more biologically active two chain forms.

Plasmin cleaves fibrin so as to release soluble fibrin degradation products (FDP's). This process also exposes carboxy-terminal lysine residues on fibrin, which increase binding of tPA and plasminogen via the affinity of kringle groups (kringle 2 on tPA and 1+4 on plasminogen) for this amino acid. This binding is impeded by lysine analogue pharmacological agents such as ε-amino caproic acid (EACA) and tranexamic acid. The recently discovered Thrombin

Activatable Fibrinolysis Inhibitor (TAFI) enzymically removes the lysine residues essential for this interaction from fibrin. TAFI is activated by thrombin when bound to thrombomodulin. The physiology of TAFI has recently been reviewed (Bajzar, 2000).

Circulating inhibitors of fibrinolysis keep a check on fibrinolytic activity. Plasminogen activator inhibitor 1 (PAI-1) inhibits the activity of tPA and uPA. Plasmin is directly inhibited by α2-plasmin inhibitor (α2-PI) and α2-macroglobulin.

A diagrammatic overview of the process of fibrinolysis is shown in figure 6.

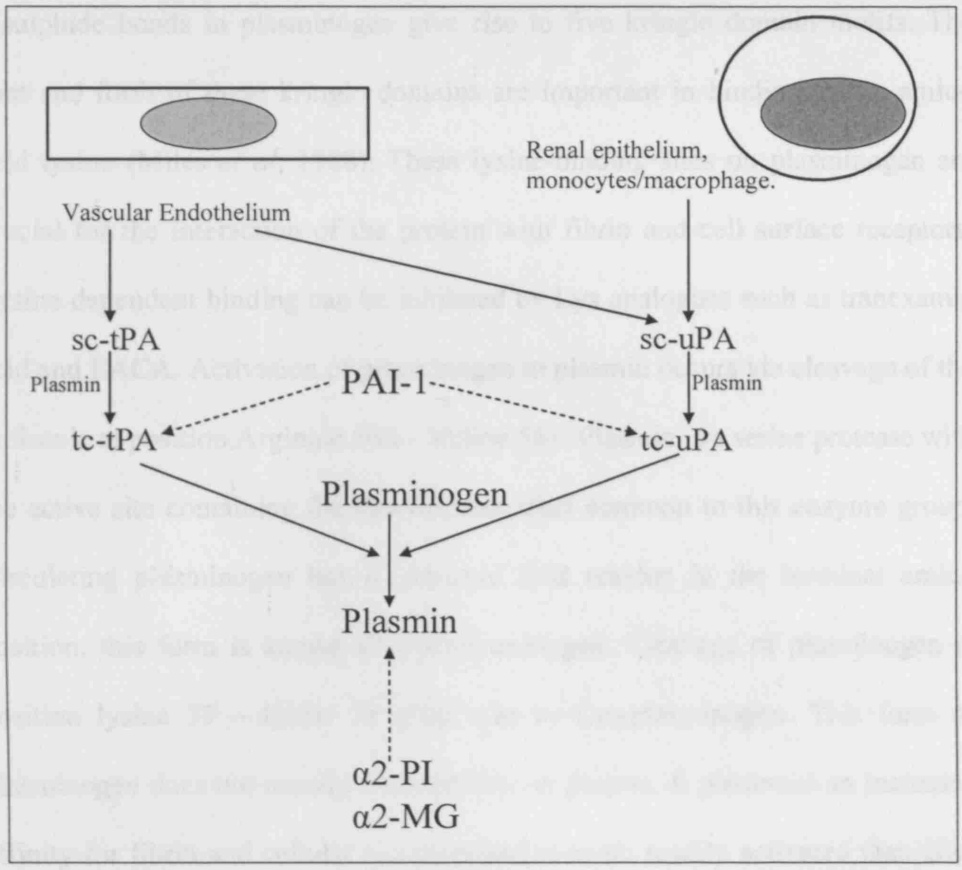


Figure 6 Basic physiology of the fibrinolytic system.

(sc = single chain; tc = two chain; α2-PI = α2-plasmin inhibitor; α2-MG = α2 macroglobulin; PAI = plasminogen activator inhibitor. Inhibition is indicated by a dashed line.

1.3.2 Proteins involved in the fibrinolytic system

In the following section, an overview of the main components of the fibrinolytic system is given. Where appropriate more detail on individual components is discussed later in the thesis.

1.3.2.1 Plasminogen

Plasminogen is primarily hepatically synthesised (Raum *et al*, 1980). The protein has a molecular weight of 92,000 Daltons and circulates at a concentration of 1.5μMol/l (138μg/ml). The gene for plasminogen is found on chromosome 6 and bears some structural relationship with the apolipoprotein a gene (part of the low density lipoprotein complex) (McLean *et al*, 1987). Sixteen

disulphide bonds in plasminogen give rise to five kringle domain motifs. The first and forth of these kringle domains are important in binding to the amino acid lysine (Miles *et al*, 1988). These lysine-binding sites on plasminogen are crucial for the interaction of the protein with fibrin and cell surface receptors. Lysine dependent binding can be inhibited by Lys analogues such as tranexamic acid and EACA. Activation of plasminogen to plasmin occurs via cleavage of the molecule at position Arginine 560 – Valine 561. Plasmin is a serine protease with the active site containing the His-Asp-Ser triad common to this enzyme group. Circulating plasminogen has a glutamic acid residue in the terminal amino position; this form is known as Glu-plasminogen. Cleavage of plasminogen at position lysine 77 – lysine 78 gives rise to Lys-plasminogen. This form of plasminogen does not usually circulate free in plasma. It possesses an increased affinity for fibrin and cellular receptors and is more readily activated than Glu-plasminogen (Wallen & Wiman, 1972);(Markus *et al*, 1979); (Hoylaerts *et al*, 1982); (Holvoet *et al*, 1985).

1.3.2.2 Plasminogen activators

Both tPA and uPA are serine proteases. TPA is a 72,000 Dalton protein. TPA is converted into a more active two chain (tc) form by cleavage at Arginine 275 – Isoleucine 276 by plasmin (Pennica *et al*, 1983). (The chains remain linked by a disulphide bond; this is also the case for tc-uPA). Both single and two chain tPA have similar plasminogen activating activity when bound to fibrin; however, two chain tPA is more active in the non-bound phase (Tate *et al*, 1987). TPA is secreted by vascular endothelial cells. There is some anatomical variation in secretion, this being greatest in the vasa vasora, pre and post capillary arterioles and venules in a study of primate and mouse tissue by immunochemistry (Levin

et al, 1997). The plasma half-life of tPA is short at about 5 minutes. This short half-life is consequent upon rapid uptake by hepatocytes and inhibition and clearance in complex with the serpin PAI-1. TPA release from endothelial cells is stimulated among other things by hypoxia, shear stress, vessel occlusion and thrombin.

Urokinase is a 54,000 Dalton protein and is expressed by vascular endothelial cells, renal epithelial cells, macrophages and some cancer cells. Tumor Necrosis Factor has been shown to increase the production of uPA by endothelial cells (van Hinsbergh *et al*, 1990). Interestingly this study showed most of the release to be on the basolateral membrane thus directing the release towards sites of peri-cellular fibrinolysis rather than towards the vascular space. The protein can be cleaved at Lysine 158 – Isoleucine 159 by kallikrein and plasmin to produce a more active two-chain form. Further plasmin cleavage of two-chain uPA results in the formation of high and low molecular weight forms consequent on the release of a 135 amino acid sequence. Only the high molecular weight form can bind the urokinase receptor (UPAR) (Cesarman-Maus & Hajjar, 2005). UPA does not bind fibrin as avidly as tPA and observed increases in plasminogen activation by uPA in the presence of fibrin may relate to conformational changes in plasminogen itself when associated with fibrin. UPA can activate plasminogen in both the fibrin bound and non-bound state (Gurewich *et al*, 1984).

Plasmin generation by sc-uPA involves three steps (Sun *et al*, 2002): 1) plasminogen activation by the intrinsic activity of sc-uPA; 2) sc-uPA conversion to tc-uPA by plasmin; 3) plasminogen activation by tc-uPA. As mentioned

previously, tc-uPA can also be formed by the enzymic action of plasma kallikrein on sc-uPA (so-called "intrinsic fibrinolysis").

1.3.2.3 Fibrin accelerates the activation of plasminogen by tPA

Recent work has revealed that there are two phases in the dissolution of a fibrin clot mediated by tPA. The first phase involves the activation of plasminogen at the surface of an intact clot while the second phase involves plasminogen binding to internal (carboxyterminal lysine residues) exposed by partial fibrin degradation. This process has been recently reviewed (Medved & Nieuwenhuizen, 2003).

1.3.2.4 Physiological inhibitors of fibrinolysis

Alpha2-plasmin inhibitor (α2-PI) is a major physiological inhibitor of plasmin. This protein belongs to the superfamily of serine proteases known as serpins. Alpha2-macroglobulin is a non-serpin circulating protein capable of plasmin inhibition, which exerts about 10% of the plasmin neutralising capacity of α2-PI. In some of the work carried out in this thesis, it was necessary to reduce the serpin activity of plasma by treatment with acetone (see chapter 4).

Of the plasminogen activator inhibitors, PAI-1 is the most abundant and is secreted by the liver, monocytes, vascular endothelial cells and platelets. Inflammation is a major stimulus to release of PAI-1. PAI-2 is synthesised in the placenta. PAI 1 and 2 both belong to the serpin superfamily.

1.3.2.5 Thrombin activatable fibrinolysis inhibitor

This protein has been the subject of a recent comprehensive review (Bouma & Meijers, 2003). This hepatically synthesised protein with carboxypeptidase activity is activated by thrombin when in complex with

thrombomodulin at the endothelial cell surface. Activated TAFI removes carboxy terminal lysine residues from fibrin (and other proteins including annexin II), thus removing the lysine dependent binding site for the kringle domains on tPA and plasminogen. Activated TAFI has a short half-life of about 10 minutes. It has recently being speculated, that reduced TAFI activation as a result of an impaired thrombin burst may contribute to the bleeding diathesis in haemophilia A. This effect may be particularly important in areas with a high fibrinolytic potential such as synovial joints.

1.3.2.6 The role of the vascular endothelial cell surface in fibrinolysis

As well as synthesising and secreting activators and inhibitors of fibrinolysis, vascular endothelial cells also provide a surface for the binding of plasminogen and its activators. This area has been the subject of recent reviews in the literature (Cesarman-Maus & Hajjar, 2005) (Hajjar & Nachman, 2001). In context, this binding capacity of endothelial cells for plasminogen and its activators is part of a system of "pericellular fibrinolysis" which is important in wound healing (Romer *et al*, 1996), macrophage tissue invasion (Falcone *et al*, 2001), tumour metastasis (Wang, 2001) and trophoblast invasion.

Using a system with Human Umbilical Vein Endothelial Cell (HUVEC) monolayers in a microtitre plate system, Hajjar *et al* demonstrated specific binding of plasminogen to HUVEC reaching a steady state over 20 minutes, which was inhibited by 70-80% in the presence of the lysine analogue ε-amino caproic acid (EACA). The binding of plasminogen to the HUVEC surface was also found to increase the catalytic efficacy of plasminogen activation by tPA by 12.7 fold compared to the fluid phase (Hajjar *et al*, 1986). Endothelial cell bound plasminogen has also been demonstrated to undergo conversion from glu-

plasminogen to the more readily activated Lys-plasminogen. Moreover, Lys-plasminogen has been detected in elulates from endothelial cells and demonstrated at the endothelial surface by immunochemistry (Hajjar & Nachman, 1988).

Further work has established that one of the endothelial binding sites for plasminogen is the protein Annexin II which can also bind tPA and therefore provide co-localisation of zymogen and activator (Hajjar, 1990) (Hajjar *et al*, 1994). Annexin II is a 40,000 Dalton protein, which binds to cell surface phospholipids in a calcium dependent manner. Purified Annexin II has been shown to increase the catalytic efficiency of tPA activation of plasminogen by up to sixty fold. Moreover, the interaction of plasminogen with Annexin II is lysine dependent being inhibited by EACA and by pre-treatment of Annexin II by carboxypeptidase resulting in the removal of carboxy-terminal lysine residues (Cesarman *et al*, 1994). (The binding of tPA to Annexin II is not impeded by these treatments). The exposure of a carboxy-terminal lysine residue probably requires cleavage of Annexin II at lys307-Arg308 by an intracellular protease (Hajjar & Krishnan, 1999). Annexin II is expressed by vascular endothelial cells, monocytes, myeloid cells and developing neuronal cells (Hajjar & Nachman, 2001). The expression of Annexin II in vascular endothelial cells is dependent on anatomical location and those in the cerebral vasculature have been demonstrated to express the highest levels (Kwaan *et al*, 2004). The physiological importance of the Annexin II plasminogen/tPA binding system has been demonstrated in animal studies. Annexin II deficient mice have been shown to exhibit reduced fibrin clearance and have increased fibrin deposition in the microvasculature (Ling *et al*, 2004); studies in the rat carotid artery have demonstrated reduction in

arterial thrombosis post Annexin II infusion (Ishii *et al*, 2001). Moreover, one hypothesis for the bleeding diathesis seen in patients with acute promyelocytic leukaemia is the over expression of Annexin II by cells of this leukaemic lineage (Menell *et al*, 1999). Lipoprotein (a) (containing apolipoprotein (a) which contains sequences homologous to kringle 4 of plasminogen) can reduce the binding of plasminogen to Annexin II; homocysteine has been shown to reduce the binding of tPA to Annexin II. These interactions have been suggested as mechanisms contributing to the pathogenicitiy of these substances *in vivo* (Hajjar & Krishnan, 1999).

At the cell surface, Annexin II is associated with the protein S100A10 (also referred to as p11) (Kassam *et al*, 1998). The proteins form a heterotetramer composed of two S100A10 molecules and two Annexin II molecules. The Waisman group have proposed that rather than directly binding to Annexin II, tPA and plasminogen are co-localised by binding to S100A10 in the heterotetramer. The role of Annexin II in this model being to anchor S100A10 to the cell surface (Waisman, 2005). Moreover, plasmin binds to S100A10 at a distinct site where it can undergo auto-proteolysis (Fitzpatrick *et al*, 2000). The Waisman group have proposed that this may be a mechanism whereby a transient pulse of plasmin activity can be generated at a cell surface (Kwon *et al*, 2005). The details of the exact nature of the binding mechanism of tPA and plasminogen to the Annexin II heterotetramer continue to be debated in the literature (Waisman, 2005) (Cesarman-Maus, 2005). A schematic of the various hypothesis involving the binding of tPA and plasminogen to Annexin II and S100A10 at the endothelial cell surface are shown in figure 7.

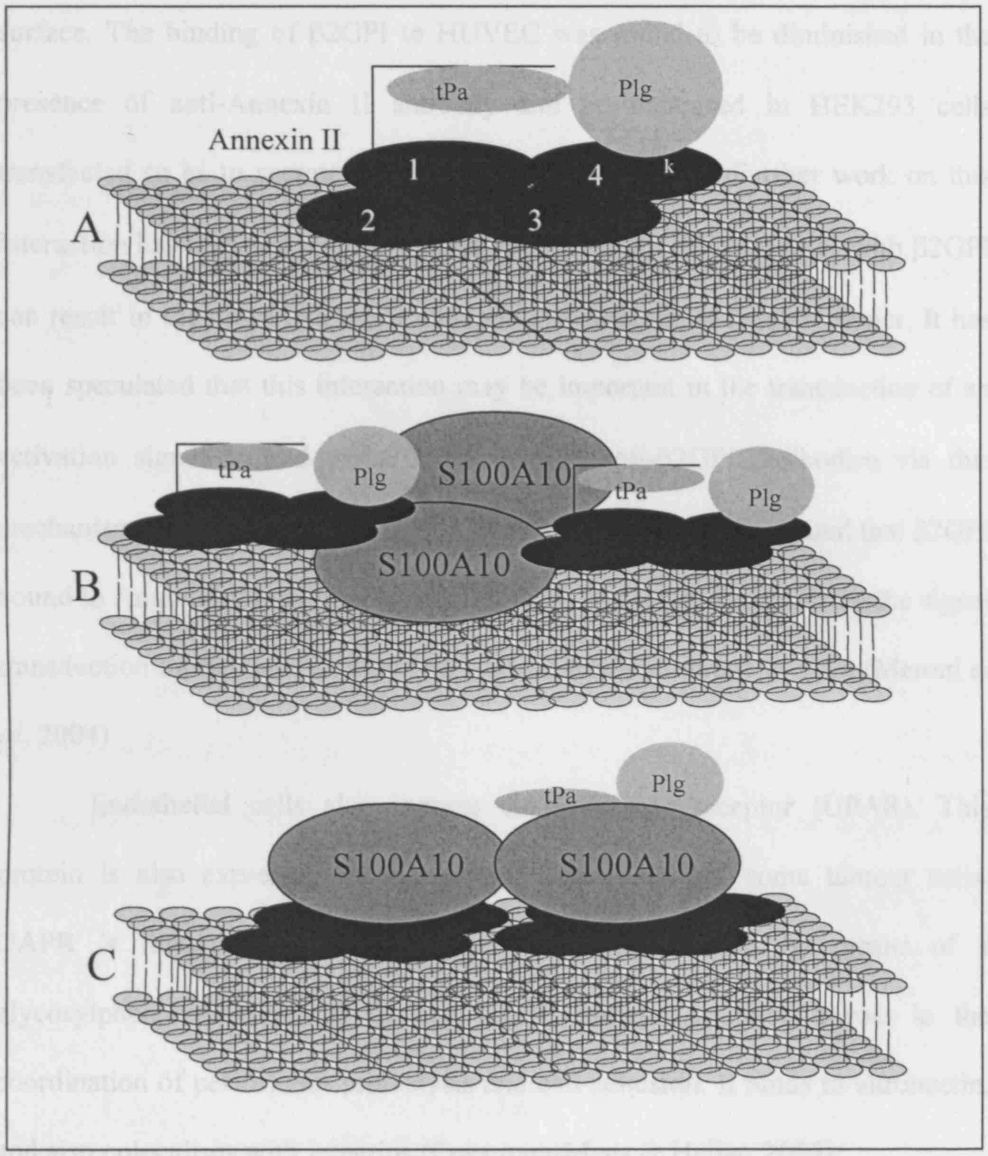


Figure 7 The binding of tPA and plasminogen to Annexin II and Annexin II heterotetramer at the endothelial cell surface.

A Plasminogen binds to carboxy-terminal lysine (k) in core repeat 4 of Annexin II. TPA binds to a LCKLSL sequence in the amino tail of Annexin II. (Core repeats of Annexin II are labelled 1-4).
B Interaction between Annexin II and plasminogen/tPA is direct but this time the heterotetramer with S100A10 is shown.
C Alternative hypothesis whereby Annexin II serves to anchor S100A10 to the cell surface and the tPA/plasminogen binding is to the latter protein.

A further layer of relevance of the Annexin II story to antiphospholipid syndrome has been afforded by recent work examining the interaction between β2GPI and Annexin II. Ma *et al* have described the interaction between β2GPI

and Annexin II as being important in the binding of β2GPI to the endothelial cell surface. The binding of β2GPI to HUVEC was found to be diminished in the presence of anti-Annexin II antibody and be increased in HEK293 cells transfected so as to secrete Annexin II (Ma *et al*, 2000). Further work on this interaction has revealed that the interaction of anti-β2GPI antibodies with β2GPI can result in the cross-linking or clustering of Annexin II heterotetramer. It has been speculated that this interaction may be important in the transduction of an activation signal to endothelial cells binding anti-β2GPI antibodies via this mechanism (Zhang & McCrae, 2005). Some workers have speculated that β2GPI bound to Annexin II may interact with the Toll like receptor explaining the signal transduction mechanism for a protein with no transmembrane domain (Meroni *et al*, 2004).

Endothelial cells also express the urokinase receptor (UPAR). This protein is also expressed by monocytes, fibroblasts and some tumour cells. UAPR is linked to the plasma membrane phospholipid by means of a glycosylphosphatidylinositol anchor. UAPR appears to have a role in the coordination of pericellular proteolysis and cell adhesion. It binds to vitronectin, and also colocalises with integrins (Cesarman-Maus & Hajjar, 2005).

1.4 Thrombophilia

Before discussing the Antiphospholipid Syndrome, it is important to place the syndrome within the wider context of conditions resulting in a predisposition to thromboembolic disease. This subject has been the focus of recent review articles in the literature (Marder VJ & Matei DE, 2001); (Lopez *et al*, 2004); (Weitz *et al*, 2004).

Virchow in 1859 first described the pathophysiological changes associated with a predisposition to venous thrombosis, namely: 1) blood stasis 2) changes in the vessel wall 3) hypercoagulability. Even in the modern setting, this triad still applies as a framework for the understanding of the prothrombotic state. Although in recent years much focus has been placed on novel acquired prothrombotic states such as the antiphospholipid syndrome and genetic predisposition to thrombosis, one must remember that other (often more common) acquired states can predispose to venous thrombosis, these are summarised in table 1.

Immobility
Tissue trauma (including surgery)
Myocardial infarction
Pregnancy and puerperium
Oestrogen therapy
Obesity
Varicose veins
Previous deep venous thrombosis
Congestive cardiac failure
Malignancy
Nephrotic syndrome
Advancing age
Hyperviscosity
Collagen vascular disease
Antiphospholipid syndrome
Myeloproliferative disease
Bechet's syndrome
Paroxysmal nocturnal haemoglobinuria
Therapy with activated clotting factors

Table 1 Acquired risk factors for venous thromboembolism. (Greaves M & Preston EF, 1999)

With regard to thrombophilia risk factors, which are commonly investigated in the haematology clinic, the antiphospholipid syndrome can manifest a much wider range of presentations than many of the other conditions seen in this context. This range of presentations is discussed in the next section. Notably however, APS results in an increased risk of both arterial and venous thrombosis. Other acquired and genetic risks for arterial thrombosis are listed in table 2.

Increased procoagulant	Fibrinogen Factor VII Factor VIII
Abnormal metabolism	Hypercholesterolaemia Diabetes mellitus Hyperhomocysteinaemia
Autoimmune disease	Antiphospholipid syndrome
Environmental	Smoking Diet

Table 2 Acquired and genetic risk factors for arterial athero-thrombosis (Greaves M & Preston EF, 1999)

With regard to venous thromboembolism many patients suffering such an event are now screened for inherited and acquired risk factors as part of a "thrombophilia screen". A list of thrombophilia risk factors which commonly make up such a screen and their respective relative risk for first episode of venous thromboembolism is shown in table 3. Within the context of Virchow's triad, the mechanisms of action of the various risks for venous thromboembolism are illustrated in table 4.

Thrombophilic defect	Estimated relative risk
Antithrombin deficiency	8-10
Protein C deficiency	7-10
Protein S deficiency	8-10
Factor V Leiden/Activated Protein C resistance	3-7
Prothrombin G20210A mutation	3
Elevated VIII (dose dependent)	2-11
Elevated IX:c	2-3
Elevated XI:c (>90th centile)	2
Hyperhomocysteinaemia	2.5
Anticardiolipin antibodies -High titre only	3.2
-All	1.6
Lupus anticoagulant	11

Table 3 Estimated relative risks for a first episode of venous thromboembolism (Weitz *et al*, 2004)

Increased baseline risk	Acute insult
Hypercoagulability	
Genetic	*Increased coagulants*
Increased coagulant: Prothrombin G20210A	Exogenous clotting factor administration
Decreased anticoagulant:	(e.g. rVIIa)
Deficiency of Protein C, Protein S, Antithrombin	*Acute anticoagulant loss*
Factor V Leiden	Nephrotic syndrome (antithrombin)
Acquired	Initial warfarinisation without heparin
Malignancy	(decreased protein C and S)
Hyperhomocysteinaemia	
Oestrogens	
Pregnancy	
Nephrotic syndrome (loss of antithrombin)	
Antiphospholipid syndrome	
Increased levels of clotting factors	
Vessel wall Changes	
Endothelial injury due to chemotherapy	Indwelling vascular catheters
Hyperhomocysteinaemia	Trauma and surgery
Vasculitis	
Antiphospholipid syndrome	
Blood stasis	
Age	Bed bound/hospitalisation
Obesity	Limb paralysis/immobilisation
Pregnancy	Right heart failure
	Long-haul flights
	Venous compression

Table 4 Potential mechanisms by which various clinical conditions can facilitate deep venous thrombosis (Lopez *et al*, 2004)

1.5 Antiphospholipid Syndrome

1.5.1 Historical

The Antiphospholipid Syndrome (APS) is a multisystem prothrombotic disorder characterised by clinical features related to thrombosis and obstetric morbidity along with the persistent production of antiphospholipid antibodies (aPL).

The first full description of this syndrome is attributed to GRV Hughes and APS is also known as Hughes' Syndrome (Boey *et al*, 1983). Over a period during the early and mid 1980's Hughes' team described patients (initially with SLE and later in a broader cohort) with a range of thrombotic, obstetric and neurological problems and positivity for serological and coagulation tests for aPL (Hughes, 1983;Harris *et al*, 1984;Hughes *et al*, 1984;Harris *et al*, 1985a;Harris *et al*, 1986). The condition was initially referred to as "anticardiolipin syndrome" (Hughes, 1985). The realisation that antiphospholipid serology could occur outside the context of connective tissue disease led to the concept of "primary" antiphospholipid syndrome as a diagnostic entity:

> "Although many of these patients fall under the heading of lupus, or lupus like disease, I believe that the group is sufficiently homogenous, and in some ways (such as the frequently negative ANA serology) sufficiently different from the typical systemic lupus erythematosis to warrant separate consideration. The manifestations of this syndrome are thrombosis (often multiple) and, frequently, spontaneous abortions (often multiple), neurological disease, thrombocytopenia and livedo reticularis." (Hughes, 1984).

While the full clinical description of APS had to wait until the 1980's, descriptions of the biological phenomena used in the diagnosis of the syndrome had been in the literature for sometime. The first description of aPL dates back to 1906 with the development of the Wasserman reaction as a test for *Treponema*

pallidum. This test employed a complement fixation reaction, with sera from patients with suspected syphilis being reacted with extracts of foetuses with congenital syphilis. In the 1940's the antigen bound in this test was found by Pangborn to be an acidic phospholipid, which would later be called cardiolipin. Large scale screening for syphilis revealed a number of patients who exhibited so called biological false positivity for syphilis. Development of other tests for treponemal disease allowed these patients to be better distinguished from patients with syphilis. Some of these false positive patients had other infections; however, some were patients with autoimmune disease (Moore & Mohr, 1952).

In the early 1950's, Conley and Hartman had reported the "lupus anticoagulant" (LA) effect of prolongation of the APTT in some severe SLE patients (Conley CL and Hartmann, 1952). Later work was to establish that patients with LA and SLE had an increased risk of thrombosis (Boey *et al*, 1983).

Work by Thiagarajan *et al* helped elucidate the mechanism of the *in vitro* LA effect. In a patient with macroglobulinaemia and LA they found the whole IgM and IgM Fab of the patient was able to impede the activation of X and prothrombin at phospholipid surfaces (Thiagarajan *et al*, 1980). Later study by Pengo *et al* revealed that IgG lupus anticoagulants could impair the binding of X and prothrombin to negatively charged phospholipid surfaces (Pengo *et al*, 1987). This work however did not explain the paradox of an *in vitro* anticoagulant effect being associated with an *in vivo* pro-coagulant effect.

Reasoning that a solid phase test for anticardiolipin should be possible to detect antibodies directed against negatively charged phospholipids (as yet a protein co-factor had not been discovered), Harris *et al* designed the first solid phase anticardiolipin (aCL) assay in 1983 (Harris *et al*, 1983). The same group

found positivity in the aCL assay to correlate with thrombosis in SLE patients. This test was found to be more sensitive than the LA tests and led to more patients with antibody activity directed against negatively charged phospholipids being found. The discovery that aCL activity in some cases cross-reacted with activity against other negatively charged phospholipids such as phosphatidylserine led to the more broad term antiphospholipid antibody (aPL) being coined (Harris *et al*, 1985b). The increased sensitivity of the aCL test however created problems due to its lower specificity. Anticardiolipin antibody activity can be detected in patients who do not have APS particularly those with infection. Moreover the spectres of standardisation of the aCL assay and which levels of antibody were pathologically significant began to raise their heads. Early work, with these problems in mind began to illustrate that IgG aCL of high titre were likely to be the most significant pattern of aCL positivity (Gharavi *et al*, 1987). In addition the first of many workshops on the standardisation of the aCL test took place in 1986 (Harris *et al*, 1987).

Another major advance in the history of the antiphospholipid syndrome came in 1990 when three separate groups elucidated that rather than binding directly to a phospholipid surface, aPL required a protein cofactor to bind to phospholipid. This cofactor was identified as β2glycoprotein I (Matsuura *et al*, 1990) (Galli *et al*, 1990) (McNeil *et al*, 1990). Other putative cofactors for aPL binding have been described since, these include prothrombin (Roubey, 1994).

1.5.2 Diagnosis of Antiphospholipid syndrome

The most widely accepted diagnostic criteria for APS derive from the international consensus statement of the 8th international symposium on antiphospholipid antibodies held in Sapporo 1998 (Wilson *et al*, 2001). These "preliminary" criteria for diagnosis are shown in table 5.

Clinical criteria

1.Vascular thrombosis

One or more clinical episodes of arterial, venous or small vessel thrombosis in any tissue or organ. Thrombosis must be confirmed by imaging or Doppler studies or histopathology, with the exception of superficial venous thrombosis. For histopathologic confirmation, thrombosis should be present without significant evidence of inflammation of the vessel wall.

2.Pregnancy morbidity

(a)One or more unexplained deaths of a morphologically normal fetus at or beyond the 10^{th} week of gestation, with normal fetal morphology documented by ultrasound or by direct examination of the fetus, or

(b)One or more premature births of a morphologically normal neonate at or before the 34^{th} week of gestation because of severe pre-eclampsia or eclampsia, or severe placental insufficiency, or

(c)Three or more unexplained consecutive abortions before the 10^{th} week of gestation, with maternal anatomic or hormonal abnormalities and paternal and maternal chromosomal causes excluded

In studies of populations of patients who have more than one type of pregnancy morbidity, investigators are strongly encouraged to stratify groups of subjects according to a, b or c above.

Laboratory criteria

1.Anticardiolipin antibody of IgG and/or IgM isotype in blood, present in medium or high titre, on 2 or more occasions at least 6 weeks apart, measured by standardised enzyme-linked immunosorbent assay for β2-glycoprotein I dependent anticardiolipin antibodies.

2.Lupus anticoagulant present in plasma, on 2 or more occasions at least 6 weeks apart, detected according to the guidelines of the International Society on Thrombosis and Hemostasis (scientific subcommittee on lupus anticoagulants/phospholipid dependent antibodies), in the following steps:

(a)Prolonged phospholipid dependent coagulation demonstrated on a screening test eg activated partial thromboplastin time, kaolin clotting time, dilute Russell's viper venom time, dilute prothrombin time, Textarin time.

(b)Failure to correct the prolonged coagulation time on the screening test by mixing with normal platelet poor plasma.

(c)Shortening or correction of the prolonged coagulation time on the screening test by addition of excess phospholipid.

(d)Exclusion of other coagulopathies eg factor VIII inhibitor or heparin as appropriate.

Definite antiphospholipid antibody syndrome is considered to be present if at least one of the clinical criteria and one of the laboratory criteria are met.

Table 5. Preliminary criteria for the classification of the antiphospholipid syndrome

After Wilson *et al* 1999. The following footnote appears in the original presentation of this classification:

"No exclusions other than those contained within the above criteria are needed. However, because of the likelihood that thrombosis may be multifactorial in patients with the antiphospholipid syndrome, the workshop participants recommend that (a) patient populations being studied should be assessed for other contributing causes of thrombosis, and (b) such populations should be stratified according to identifiable or probable risk factors, e.g. age or comorbidities. Specific limits were not placed on the interval between clinical event and the positive laboratory findings. However, it was the view of many at the workshop that (a) information about such intervals should be assessed when relevant, and (b) the relatively strict laboratory criteria (including the requirement that results be positive on repeat tests performed at least six weeks after the initial test) would help exclude antiphospholipid antibody positivity that represents an epiphenomenon to the clinical events."

The main purpose of these criteria was to focus on a diagnosis of "definite" APS rather than "possible" APS. This was in order to facilitate the use of the classification in study of treatment and pathogenic mechanisms of APS. The classification therefore does not include some clinical conditions which although seen in patients with APS do not have so strong an association with the syndrome as the clinical features mentioned in the classification. Such features include thrombocytopenia, haemolytic anaemia, transient ischaemic attacks, transverse myelopathy/myelitis, livedo reticularis, cardiac valve disease, chorea and migraine. Another criticism of this classification is that in the section on serology, it omits mention of anti-β2GPI activity (and also anti-prothrombin and anti-phosphatidylethanolamine activity).

Recently (Miyakis *et al*, 2006) an updated international consensus statement on the classification of APS has been published. Importantly, this revision now includes anti-β2GPI activity as part of the diagnostic criteria for the syndrome. In addition, the lag time between tests has been extended to 12 weeks and a recommendation has been added that no more than 5 years between clinical events and serological positivity should elapse for a diagnosis to be made.

1.5.3 Clinical risk associated with the presence of aPL

Two recent systematic reviews of the literature have attempted to stratify the risk of thrombotic events associated with the presence of different markers of APS (Galli *et al*, 2003b;Galli *et al*, 2003a). These studies examined the literature between 1988 and 2000 looked at data on aCL and LA from over 4000 patients and 3000 controls. In these studies no attempt at meta-analysis was made because of heterogeneity between studies in terms of design, clinical end points

and laboratory tests. The presence of LA was found to be the strongest predictor of thrombosis with an odds ratio for thrombosis of 5-16 times controls in individuals with persistent LA positivity. The best association for thrombosis and aCL was found in this study when arterial events and IgG class aCL of titre 33-40 GPLU were considered. The association of LA and thrombosis was found irrespective of the method of detection, the type and site of the event and the presence of SLE. A further analysis by this group concentrated on anti-β2GPI and anti-prothrombin antibodies. The authors of this analysis point out that because of the association between SLE, LA and aCL with anti-β2GPI and anti-prothrombin activity, it is difficult to determine whether these antibody activities are independent risk factors for thrombotic events. The best association between anti-β2GPI antibodies and thrombosis was with the IgG class. However the authors mention in their conclusions that most of the data in this study on anti-β2GPI antibodies came from retrospective studies with only a few using multivariate analysis. Galli *et al* mention in both of these systematic reviews that an important goal for the future must be to standardise assays for aPL to facilitate future "cross pollination" between studies.

1.5.4 Pathogenic mechanisms in the Antiphospholipid syndrome

While the serological and clinical classification of APS has been broadly speaking agreed upon for some time, the basic science that underlies the pathogenesis of APS is still yet to be fully understood. Even at a casual glance, the syndrome throws up the conundrum of an *in vitro* anticoagulant phenomenon, which has a strong association with thrombosis *in vivo*. The first published evidence that aPL could be directly pathogenic came in 1981 (Carreras *et al*, 1981). This group demonstrated that the IgG fraction from a patient with lupus anticoagulant could reduce the release of prostacyclin from rat aorta rings.

Along with an understanding that aPL may be directly pathogenic at a cellular level, another paradigm shift in our understanding of the pathogenesis of APS came with the discovery that aPL were directed against proteins bound to negatively charged phospholipid rather than phospholipid directly. The first protein found to be a cofactor for aPL was β2-glycoprotein I (β2GPI) (Galli *et al*, 1990) (McNeil *et al*, 1990) (Matsuura *et al*, 1990), the detailed biochemistry of β2GPI is discussed in a separate section. Some lupus anticoagulants are also directed against prothrombin in complex with phospholipid (Bevers *et al*, 1991). Many different theories have been presented in an attempt to explain the pathology of APS. In the following section some of these will be discussed. A more complete discussion relating to possible interactions between aPL and the fibrinolytic system is presented later.

Not all patients with aPL positivity will develop the signs and symptoms of APS. In a study by the Italian aPL registry, patients with either positive LA or aCL followed up for 3.9 years 34 of 390 patients developed a thrombosis (Finazzi *et al*, 1996). A previous history of thrombosis increases the risk of

recurrence, which may be as high as 20%/patient year (Khamashta *et al*, 1995). As well as a previous event, a significant interplay between aPL and other risk factors for both venous and arterial thrombotic events is evident. In a study of over 400 patients with APS, it was demonstrated that at the time of thrombosis 50% of patients had coincident risk factors (Giron-Gonzalez *et al*, 2004). The concept of a "two-hit" hypothesis for pathogenic events in APS has developed following observations of this kind.

I feel that it is important to mention that while many different mechanisms for the features seen in APS have been published, they are not necessarily mutually exclusive and it may well be that many different mechanisms are at work in this complex disease. A list of possible pathogenic mechanisms in APS is listed in table 6.

Possible pathogenic mechanisms in the antiphospholipid syndrome:
Impairment of the action of activated protein C
Interference with the Annexin V "anticoagulant shield"
Induction of increased endothelial and monocyte tissue factor
Induction of increased expression of endothelial cell adhesion molecules
Impairment of fibrinolysis
Increased platelet activation and disruption of platelet eicosanoid metabolism
Interaction with the contact system

Table 6 Possible pathogenic mechanisms of APL leading to a procoagulant state

1.5.4.1 Protein C inhibition

Activated protein C and its cofactor protein S must assemble at an anionic phospholipid surface in order to exert its effect of inactivating factors Va and VIIIa. Purified IgG and IgM from patients with APS have been shown to inhibit the inactivation of Va by activated protein C at the surface of vascular

endothelial cells *in vitro* (Borrell *et al*, 1992). Some limited evidence is available that aPL may be directed against thrombomodulin (the protein which thrombin binds to in order to activate protein C) (Oosting *et al*, 1993). A further layer of interaction between APS and the protein C system is provided from evidence that β2GPI can, under certain conditions, inhibit the phospholipid binding of activated protein C (Mori *et al*, 1996). In one study of 22 APS patients, IgG autoantibody activity against proteins C and S were found in 4 and 12 patients respectively (Pengo *et al*, 1996). Antibody activity against EPCR has also been demonstrated in a cohort of patients with unexplained fetal death and aPL and found to be an independent risk factor for such events (Hurtado *et al*, 2004).

The presence of aPL has also been found to cause an acquired activated protein C resistance (APCR) phenotype in some patients. In a study of 96 patients with SLE, Nojima *et al* found that 33 of the patients had acquired APCR and that the presence of this was significantly higher in patients who had suffered venous thrombotic complications (Nojima *et al*, 2002). This study found using multivariate logistical regression that the presence of acquired APCR was significantly associated with the presence of LA and anti-prothrombin antibody activity together. A cross-sectional cohort study in 59 children with SLE found that acquired APCR was significantly associated with LA activity (but not aCL) and that acquired APCR was significantly associated with thromboembolism in this cohort (Male *et al*, 2001). A later study in adult SLE patients found acquired APCR to be significantly associated with both the presence of aPL and increased risk for arterial thrombosis and pregnancy morbidity (Munoz-Rodriguez *et al*, 2002). The exact mechanism of the acquired APCR seen in association with aPL has still to be elucidated, a number of studies have demonstrated that aPL can

inhibit the binding of protein C and S to negatively charged phospholipid surfaces which would explain the phenomena of APCR observed (Malia *et al*, 1990) (Marciniak & Romond, 1989) (Nojima *et al*, 2002). Another possible explanation for APCR in APS is antibody activity directed against factor V. This has been described in a case report only (Kalafatis *et al*, 2002).

1.5.4.2 Annexin V

Annexin V is a protein which associates with negatively charged phospholipid surfaces in a calcium dependent manner. It is believed this allows annexin V to act as a barrier at procoagulant surfaces to the binding of procoagulant protein complexes. This may be physiologically most important in the placental circulation, indeed the infusion of anti-annexin V antibodies can reduce fetal viability in mice (Wang *et al*, 1999). The importance of annexin V as an auto antigen in APS is debatable. There is data available that patients with APS manifest anti-annexin V activity (Lakos *et al*, 2000). Arnold *et al* also found an increased incidence of anti-annexin V antibodies in aPL positive women but this was not found to be an independent marker for recurrent miscarriage (Arnold *et al*, 2001). Similarly, Ogawa *et al* described an increase in anti-annexin V antibodies in individuals with APS although again their presence did not associate significantly with clinical manifestations of the syndrome (Ogawa *et al*, 2000).

1.5.4.3 Increased expression of monocyte tissue factor

That exposure of monocytes to aPL can lead to increased surface expression of tissue factor and therefore an increased procoagulant potential has been known for more than ten years (Kornberg *et al*, 1994). Recently, this

phenomenon has been further characterised. The exposure of monocytes to purified aPL has been shown to induce monocyte Tissue Factor mRNA at two hours and lead to an increase in surface expression of the molecule at six hours. Moreover, this process has been shown to be inhibited pharmacologically by dilazep (an adenosine uptake inhibitor, which has also been shown to reduce TNF alpha induced Tissue Factor upregulation) (Zhou *et al*, 2004).

1.5.4.4 Endothelial cell activation

The vascular endothelium is intimately involved in the regulation of the coagulation mechanism and fibrinolysis. Antiphospholipid antibodies are known to interact with the surface of endothelial cells (which bind and may express β2GPI) and recent work has shown that this interaction can lead to changes in the physiology of the endothelium. Much of the research in this area has concentrated on the up-regulation of cell adhesion molecules (CAMs), which are expressed at the luminal surface of the endothelial cells.

The immunoglobulin from patients with APS have long been known to bind to the surface of vascular endothelial cells. McCrae *et al* reported increased immunoglobulin binding to HUVEC in culture by sera samples from patients with aPL positivity compared to normal controls (McCrae *et al*, 1991). Previously, the inference that aPL interacted with components of the endothelial surface had been made. This was based on the evidence that aPL could modulate some endothelial associated phenomenon such as thrombomodulin dependent protein C activation and prostacyclin release (Carreras & Vermylen, 1982;Freyssinet *et al*, 1986;Cariou *et al*, 1988). The binding of aPL to the endothelial surface is believed by most authors to be mediated via binding to β2GPI at the endothelial cell surface. B2GPI has been shown to bind to the EC

surface by means of domain V (Del Papa *et al*, 1998). B2GPI may also associate with other proteins at the EC surface. As discussed later, the pro-fibrinolytic protein annexin II may provide a binding site for β2GPI at the EC surface (Ma *et al*, 2000). The source of β2GPI found at the surface of EC has also been debated. B2GPI has been demonstrated in HUVEC at the protein and mRNA level (Caronti *et al*, 1999), however a different group produced results at odds with these findings (Alvarado-de la Barrera *et al*, 1998). The failure of some cell activation experiments in serum free media (see later) would also indicate that EC are not a major source of the β2GPI found at their surface and that the source of the molecule is plasma.

By the mid 1990's, evidence began to emerge that in addition to possible interference with processes at the cell surface, that aPL binding to endothelial cells could also lead to signal transduction events resulting in a pro-inflammatory phenotype. Simantov *et al* reported that IgG purified from patients with aCL activity caused an increase in ICAM-1, VCAM-1 and E-selectin expression. This was paralleled by an increase in monocyte adhesion. This effect was not seen when cells were incubated with IgG in serum free conditions although it could be restored by the addition of β2GPI to the system (Simantov *et al*, 1996). Later work by Pierangeli *et al* served to quantify this increase in CAM expression using a cyto-elisa method. This group exposed HUVEC to 100μg/ml of cardiolipin purified IgG from patients with APS. They also examined leucocyte adhesion and thrombus formation in the cremaster muscle of aPL exposed mice: an increase in both parameters was observed in the aPL exposed tissue (with one exception out of six) compared to that exposed to normal IgG (Pierangeli *et al*, 1999). This effect was found to be diminished in a model utilizing CAM

deficient mice (Pierangeli *et al*, 2001). A study examining the levels of soluble ICAM-1, VCAM-1 and E selectin found increased levels of soluble VCAM-1 in the sera of patients with primary and secondary APS. Soluble VCAM-1 levels were higher in APS patients with severe recurrent thrombosis compared to other APS patients (Kaplanski *et al*, 2000). In contrast, Frijns *et al* reported no increase in soluble VCAM-1 or P-selectin in SLE patients sub divided on the basis of LA positivity and thrombotic history. In this study an increase in these parameters was seen when the SLE patients were compared with healthy controls. The study did note a reduction in serum thrombomodulin levels in the patients with both LA and thrombosis compared to those without (Frijns *et al*, 2001).

IgG anti-β2GPI has also been demonstrated to increase endothelial Interleukin–6 and prostaglandin F1-alpha secretion (Del Papa *et al*, 1997). Cultured endothelial cells in which an experimental "wound" is created by scraping away a portion of the cells, have been shown to exhibit reduced migration and proliferation in the presence of endothelial reactive aPL (Lanir *et al*, 1998). The expression of tissue factor by endothelial cells has also been shown to increase following exposure to monoclonal IgG anti-β2GPI (and $F(ab)_2$ fragment) derived from a patient with APS (Kornberg *et al*, 2000). Exposure of HUVEC in culture to aPL (from patients and monoclonal derived) has also been shown to induce increased production of Monocyte Chemoattractant Protein I (MCP I). The workers involved in this study also found that levels of MCP I correlated with aCL titres in SLE patients (Cho *et al*, 2002).

The activation of endothelial cells by aPL may be open to pharmacological manipulation. This opens up the tantalising prospect of treatments for APS that do not necessitate anticoagulation. Meroni *et al* have

reported that Fluvastatin (an HMG CoA reductase inhibitor) can inhibit the development of an activated phenotype in endothelial cells exposed to aPL (Meroni *et al*, 2001). The inhibition of E-selectin expression correlated with reduced binding of the transcription factor NF-kB (which recognises multiple sites in the E-selectin promoter region) to DNA. Statin class drugs can also reduce the inflammatory actions of TNF-alpha and lipopolysaccharide on endothelial cells and this activity seems independent of their lipid lowering actions.

Later work has confirmed the importance of the NF-kB system in the signal transduction pathway leading to endothelial cell activation following exposure to aPL. Dunoyer-Geindre *et al* have demonstrated an increase in NF-kB translocation to the nucleus on exposure of HUVEC to polyclonal rabbit anti-β2GPI antibodies and monoclonal anti-β2GPI antibodies *in vitro*. This effect was much slower in the aPL treated cells than that observed on stimulation with TNF-alpha (Dunoyer-Geindre *et al*, 2002).

Further work on the pathway involved in endothelial activation by aPL has involved the use of transfected HMEC-1 cells with dominant-negative constructs of TRAF6 and MyD88. This work has shown that these messengers (but not TRAF2) are needed upstream of NF-kB translocation to induce endothelial activation in the presence of aPL (Raschi *et al*, 2003). These results strongly implicate the Toll Like Receptor (TLR) family in the signal transduction of aPL binding to an endothelial cell surface. The Toll Like Receptors are part of the innate immune response and bind microbial components including lipopolysaccharide. This would mean that aPL use the same signaling molecules as lipopolysaccharide and Il-1. The authors speculate as to whether the presence

of amino acid sequences in β2GPI which are common to some microbial pathogens (Blank *et al*, 2002) could explain why the protein in the presence of aPL may associate with TLR's. There has also been speculation in the literature as to whether aPL/β2GPI/annexin II complexes could interact with the TLR at the EC surface (Meroni *et al*, 2004).

1.5.4.5 Dysregulation of prostaglandin and prostacyclin synthesis

Changes in the synthesis of prostacyclin and thromboxane A2 by endothelial cells and platelets respectively have been described in the context of APS. A study in the early 1990's analysing the urinary excretion of metabolites of eicosanoids found an increase in excreted 11-dehydro thromboxane B2 with a much smaller increase in urinary 2, 3-dinor-6-keto-prostaglandin F1alpha. These results suggesting an imbalance in the equilibrium of prostacyclin and thromboxane A2 with the balance in favour of platelet activation (Lellouche *et al*, 1991). Additionally, phospholipase A_2 activity has been shown to be reduced in endothelial cells stimulated with thrombin (or calcium inophore A23187) in the presence of aPL (Schorer *et al*, 1992).

1.5.4.6 Increased platelet activation

Increased platelet activity has been suggested as the mechanism of increased thrombosis in APS. That aPL can bind to platelet surfaces has been demonstrated (Shi *et al*, 1993a). Font *et al* have described an increase in platelet – subendothelium interaction under flow conditions in the presence of monoclonal aPL and β2GPI (Font *et al*, 2002). The thrombin receptor agonist peptide induced increase in platelet surface CD41a and CD61 has been shown to

be increased in the presence of aPL. Moreover, this effect has been shown to be abrogated by the presence of hydroxychloroquine (Espinola *et al*, 2002).

1.5.4.7 Interactions between the contact system and antiphospholipid antibodies

Antibody activity against factor XII has been demonstrated in the immunoglobulin fraction of patients with lupus anticoagulants (Jones *et al*, 1999). The authors of this study postulated as to whether this activity related to the lower activities of factor XII observed in the plasma of APS patients and whether this activity could lead to inhibition of the profibrinolytic properties of factor XIIa. The same group has also reported anti-XII activity in female APS patients with recurrent fetal loss (Jones *et al*, 2001). Interestingly β2GPI has been reported to competitively inhibit the activation of prekallikrein by factor XIIa (Schousboe, 1988). The same group has also reported that both β2GPI and anti-β2GPI-β2GPI immune complex may inhibit the autoactivation of XII in the presence of phospholipid (Schousboe & Rasmussen, 1995). The authors suggest that the mechanism here may be one of competition for negatively charged phospholipid between the anti-β2GPI immune complex and factor XII, a similar mechanism to that now believed to cause the *in vitro* lupus anticoagulant effect. Previously β2GPI had been shown to reduce the kaolin induced activation of XII (Henry *et al*, 1988).

Moreover, autoantibody activity has been demonstrated against kininogen bound to phosphatidylethanolamine (PE) in patients with anti-PE activity (Sugi & McIntyre, 1995). This recognition may however be due to epitopes exposed as a result of kininogen-PE binding which are not exposed when kininogens bind to other negatively charged phospholipids (Sugi & McIntyre, 1996). It has been speculated that disruption of the kallikrein – kinin system in the utero-placental

unit by some APL could result in early pregnancy failure in patients with APS (Sugi & Makino, 2000).

1.5.4.8 Impaired fibrinolysis

The experimental work in chapter 5 of this thesis will concentrate on the interaction between aPL and the fibrinolytic system. A review of the current literature in this area is presented in the introduction to chapter 5.

1.6 Beta2-glycoprotein I

Beta2-glycoprotein I (β2GPI) is probably the most extensively studied protein in the context of cofactor activity for antiphospholipid antibodies. In this section a discussion of the structure, function and interaction of the protein with aPL will be presented.

In 1990 evidence emerged that the binding of aPL to cardiolipin was mediated by a cofactor found in plasma. The source of this cofactor in the aCL assay is the bovine serum used in the blocking phase of the test. Three groups published data in 1990 illustrating that β2GPI (also known as apolipoprotein H) was a cofactor which could facilitate aPL binding to negatively charged phospholipids (Galli *et al*, 1990;McNeil *et al*, 1990;Matsuura *et al*, 1990).

1.6.1 Structure of β2GPI

β2GPI is a 50 kDa glycoprotein consisting of 326 amino acids (Lozier *et al*, 1984). The gene for β2GPI is located on chromosome 17 at position q23-24, the gene contains eight exons and is 18 KB in length (Okkels *et al*, 1999). β2GPI is mainly synthesised in the liver (Steinkasserer *et al*, 1991). The protein has 5 domains the first four of which show homology with complement control protein repeats (Schwarzenbacher *et al*, 1999). These domains have been variously referred to as "sushi" domains, short consensus repeats and complement control protein repeats. The protein is highly glycosylated, its carbohydrate content comprises some 19% of the total molecular weight of 50,000 Daltons (Gambino *et al*, 1999). The domains are referred to as domains I-V with domain I being at the amino terminal end. As shown in figure 8, the overall structure of β2GPI resembles a J-shape or "hockey stick" with domain V forming the tail of the

structure. Synthesis of β2GPI is predominately hepatic, however, endothelial cells have also been shown to express the protein (Caronti *et al*, 1999) although with regard to endothelial synthesis this finding is not universally reproducible (Alvarado-de la Barrera *et al*, 1998). The protein has a plasma concentration of approximately 200 μg/ml.

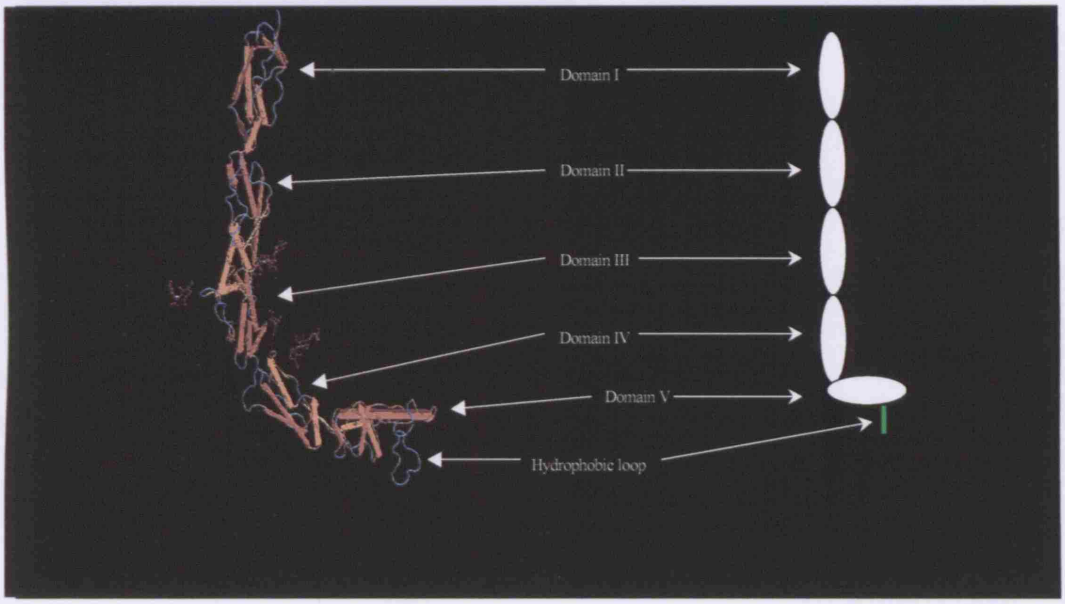


Figure 8 The structure of β2-glycoprotein I

Illustration of the structure of β2GPI in worm format to the left and with a simplified diagram to the right illustrating the domain structure and the position of the hydrophobic loop in domain V. (Structure diagram generated at the Entrez-structure website).

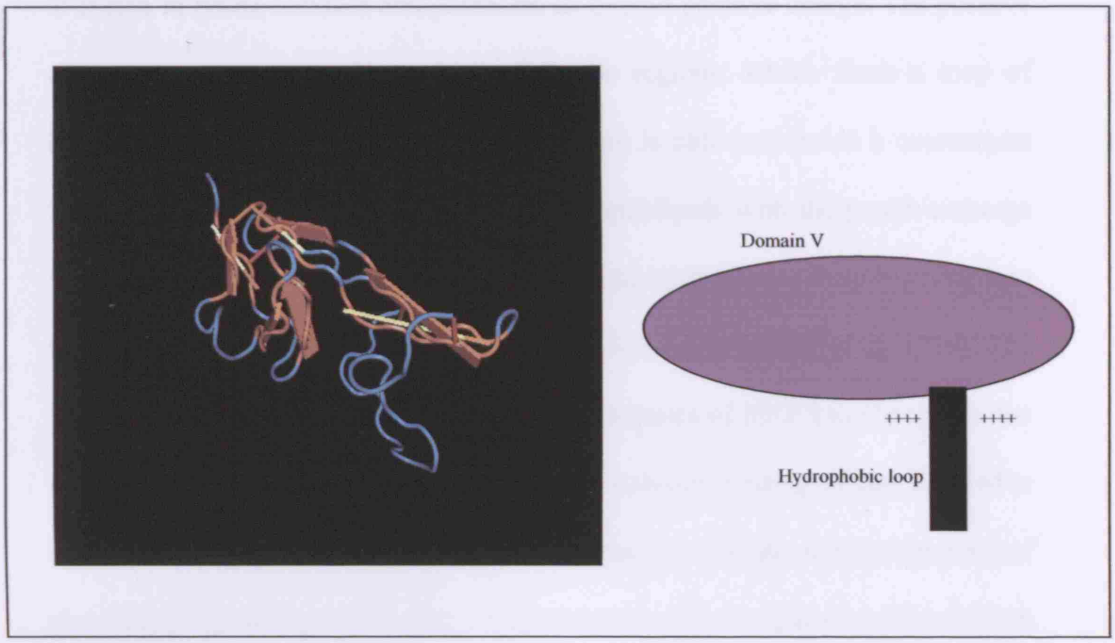


Figure 9 Structure of domain V of β2GPI

Figure illustrating the position of the hydrophobic loop and positive charge clusters in domain V critical for the interaction of the protein with negatively charged phospholipid surfaces. (Structure diagram generated at the Entrez-structure website).

β2GPI contains motifs in its fifth domain, which are crucial to the ability of the protein to interact with negatively charged phospholipid surfaces. Domain V is rich in lysine residues and possesses an overall positive charge. The positive charge clustering is most pronounced in the regions, which flank a loop of hydrophobic residues (313-316). This structure is able to provide a counterpart for interactions with negatively charged phospholipids with the positive charge interacting with the phospholipid surface and the hydrophobic loop inserting into the hydrophobic inner layer of phospholipid (Schwarzenbacher *et al*, 1999). The sensitivity of the phospholipid binding characteristics of β2GPI to changes in the structure of domain V are illustrated by the reduced binding of the Trp316Ser and Cys306Gly polymorphic variants of the protein and the reduced binding of the protein following cleavage of its hydrophobic loop by plasmin (all of which are discussed later in this thesis in more detail). There is some evidence that β2GPI may take on a "flat" as well as an upright position at negatively charged phospholipid surfaces (Wang *et al*, 2002).

1.6.2 Physiological roles of β2GPI

β2GPI is found in human plasma at a concentration of approximately 200 μg/ml; much of the circulating form of the protein is associated with chyclomicrons and other lipoproteins (Polz *et al*, 1979;Polz & Kostner, 1979). β2GPI is also found in the renal parenchyma, and in spite of its large size it is filtered by the glomerulus and is subsequently reabsorbed by the renal tubular system (β2GPI can be used as a marker of renal tubular disease) (Klaerke *et al*, 1997).

As well as having a role in the autoimmune response seen in patients with APS, β2GPI has also been found to be physiologically active in haemostasis and some aspects of lipid homeostasis. The protein can reduce the ADP induced aggregation of platelets (Nimpf *et al*, 1985). β2GPI has been shown to interfere with the binding of prothrombinase complex to platelets and phospholipid vesicles (Nimpf *et al*, 1986). β2GPI has also been reported to interact with the platelet IIb/IIIa receptor in the context of platelet microparticle membranes (Vallar *et al*, 2001). This interaction was however at the level of the immunoreactivity of the receptor complex and did not appear to have an effect on fibrinogen binding.

The protein has also been demonstrated to inhibit the kaolin induced activation of XII and the activation of the contact system in the presence of ellagic acid-phospholipid suspension (Henry *et al*, 1988;Schousboe, 1985). In addition, β2GPI has been demonstrated to inhibit Xa production at platelet surfaces; this effect can be abrogated in the presence of aPL (Shi *et al*, 1993b). It is thus possible, that β2GPI may act as a "gatekeeper" for negatively charged phospholipid sites and regulate binding of procoagulant complexes to these. Interestingly, as with other natural anticoagulant proteins (such as protein C and antithrombin), plasma β2GPI levels fall in individuals with extensive thrombosis or DIC (Brighton *et al*, 1996). In contrast to these anticoagulant roles, β2GPI can inhibit the inactivation of Va by protein C in the presence of limiting concentrations of phospholipid (Mori *et al*, 1996).

Analysis of thrombin generation *in vitro* in plasma samples from β2GPI null mice reveals a reduction in thrombin generation in mice not expressing β2GPI compared to those producing the protein (Sheng *et al*, 2001). This study

also demonstrated a reduced incidence of survival of β2GPI null fetus to term over that expected by Mendelian calculation. This effect was only seen when looking at the offspring of β2GPI null/normal heterozygote mice and not in the offspring of null/null homozygote mice. The authors of the study speculate whether this reflects a selective disadvantage for β2GPI null fetus in a β2GPI expressing mother. In contrast to this Murine study, analysis of coagulation (thrombin generation, response to activated protein C and euglobulin clot lysis time) in two human β2GPI deficient individuals showed normal results (a slight shortening of the DRVVT was noted) (Takeuchi *et al*, 2000). In addition, β2GPI deficiency has not been demonstrated to be a risk factor for thrombosis (Bancsi *et al*, 1992).

B2GPI may also have a role in the inhibition of the development of atherosclerosis. The protein has been shown to bind to and inhibit the uptake of oxidised lipoproteins by macrophages. Monoclonal aCL were found to interfere with this process (Hasunuma *et al*, 1997). B2GPI can also increase the activity of lipoprotein lipase (Nakaya *et al*, 1980).

1.6.3 How do aPL bind to and interact with β2GPI?

Central to any effort to understand the pathophysiology of APS must be an understanding of the interaction between the autoantibodies seen in the condition and the way in which they interact with their target antigens. While this section will concentrate on β2GPI, it must be borne in mind that β2GPI is unlikely to be the only target antigen in APS and also that pathological processes in the condition may be due to β2GPI interacting with other proteins in concert with aPL. In terms of clinical importance however, anti-β2GPI activity in some

studies correlates well with clinical manifestations of APS (McNally *et al*, 1995;Tsutsumi *et al*, 1996). A recent review (Galli *et al*, 2003a) showed that in retrospective analysis of previous studies while most showed an association between anti-β2GPI activity and thrombosis, because most studies involve patients with SLE, aCL activity or LA activity, it is difficult to establish the value of anti-β2GPI antibodies as an independent risk factor for thrombosis.

The detailed nature of the interaction of aPL and β2GPI has also been the subject of debate in the literature. The possibilities for interaction include aPL binding a β2GPI-phospholipid complex, a cryptic epitope that is "opened up" when β2GPI binds phospholipid (or a high binding ELISA plate (Matsuura *et al*, 1994)) or aPL binding to a high density of β2GPI at a negatively charged phospholipid surface. The site of the main autoimmune epitope on β2GPI has also been a matter of difference between authors. The most convincing evidence indicates that domain I of β2GPI contains the major antibody binding sites of the molecule although data is available to support aPL binding to other regions of the protein. These subjects have recently been reviewed (Giles *et al*, 2003b). Analysis of the gene sequences of IgG monoclonal anti-β2GPI antibodies reveals that specific binding to β2GPI is the result of antigen-driven somatic mutation. The accumulation of arginine, asparagine and lysine residues in the IgG complementary determining region appears particularly important in conferring antibody specificity (Giles *et al*, 2003a).

How the binding of aPL to their target protein/phospholipid complexes lead to the pathological changes seen in APS is still under research. The dimerisation of β2GPI following binding of aPL may explain the lupus anticoagulant phenomena *in vitro*. Work by Lutters *et al* using genetically

engineered β2GPI in a dimerised form (apple II-β2GPI) has shown that this is able to reproduce LA activity (Lutters *et al*, 2001). Dimerised β2GPI has an increased affinity for negatively charged phospholipid surfaces and may interfere with the assembly of procoagulant protein complexes thus producing an anticoagulant effect *in vitro*.

A discussion of the physiology and functional implications of proteolysis of domain V of β2GPI is presented in the introduction to chapter 4. Details of genetic polymorphisms in β2GPI are presented at the start of chapter 6.

1.7 Other cofactor proteins for aPL binding

Of all the potential cofactor proteins for aPL binding to phospholipid, after β2GPI, prothrombin is probably the most extensively studied. While the possibility that prothrombin could be a target for aPL was raised in the 1950's (Loeliger, 1959), definitive evidence for anti-prothrombin activity in APS was not available until the 1980's. In 1983 the presence of non-neutralising anti-prothrombin antibodies (anti-PT) was described in patients with LA and hypoprothrombinaemia (Bajaj *et al*, 1983), a year later prothrombin-Ig complexes were demonstrated in the plasma of patients with LA but normal prothrombin levels (Edson *et al*, 1984). The subject of anti-PT activity in APS has been the subject of a recent review (Galli & Barbui, 1999). The clinical significance of anti-PT detection has been the subject of many papers in the literature. A recent systematic review of many of these has recently been performed. This extensive review found that in eight studies of anti-PT with

multivariate analysis, two showed that anti-PT was an independent risk factor for thrombosis. Three studies demonstrated anti-PT adding to the risk associated with the presence of LA or aCL positivity. The authors of this systematic review suggested that more studies were needed to firmly establish the risk associated with anti-PT activity (Galli *et al*, 2003a).

As discussed in the section on pathogenic mechanisms in APS, antibody activity against several other potential protein cofactors for aPL binding have been found. These include components of the contact system (Jones *et al*, 1999), annexin V (Arnold *et al*, 2001), annexin II (Cesarman-Maus *et al*, 2006) and the protein C and S system (Pengo *et al*, 1996). A more detailed consideration of the interaction of aPL with components of the fibrinolytic system is given in the introduction to chapter 5.

1.8 Aims of this thesis

As discussed in this general introduction, there are many potential pathogenic mechanisms to explain the clinical manifestations of the antiphospholipid syndrome. I was particularly interested to further explore the pathophysiology of APS with a particular focus on the fibrinolytic system.

The pathophysiology of APS potentially intersects at two major points with fibrinolysis. One of these is at the level of interference of aPL with the clot clearance process. I was especially interested to see whether any interaction takes place between aPL and the binding of fibrinolytic components at cellular surfaces. APL and plasminogen are both known to bind to endothelial cell surfaces and I therefore examined this area of the fibrinolytic process. In addition I wanted to test whether aPL could interfere with fibrinolysis in a more global test of fibrinolysis such as the plasma clot lysis assay. The APS and fibrinolysis also intersect at the level of proteolytic processing of β2GPI by plasmin (in domain V of β2GPI). I wanted to test whether aPL could interfere with the processing of β2GPI by plasmin and also see whether genetic polymorphisms in domain V of the protein could affect this process. Other proteolytic enzymes have also been shown to specifically cleave domain V of β2GPI and I also wanted to see whether plasma kallikrein and factor Xa had a similar action using techniques developed in the first part of this work.

An interest in domain V of β2GPI led me to investigate one of the stable polymorphisms seen in this region of the protein – the Cys306Gly polymorphism. I was interested to see whether this non-phospholipid binding form of β2GPI had a protective effect against the production of aPL. In addition

this work allowed me to identify patients with β2GPI variants useful for the investigation of the interaction of domain V of β2GPI and plasmin.

In order to carry out the work discussed above I needed to recruit patients for study from our cohort of APS patients at UCLH. To help in this process I collated serological and clinical data on the cohort at UCLH. This process allowed me during my period of study to test a newly proposed serological classification of APS and this became a secondary aim of the thesis.

Specific aims

1) To examine the pattern of serological positivity for aPL in our patient cohort at UCLH.
2) To determine whether aPL can impede the proteolytic action of plasmin on β2GPI.
3) To investigate other proteolytic enzymes for activity against domain V of β2GPI.
4) To examine the effect of domain V polymorphisms in β2GPI on the proteolysis of the protein by plasmin.
5) To examine the effect of the Cys306Gly polymorphism on the production of aPL.
6) To investigate whether aPL can modulate plasminogen binding to cellular surfaces.
7) To test whether aPL can interfere with fibrinolysis in a global fibrinolytic assay.

Chapter 2 General Methods

2.1 Sample collection and storage

2.1.1 Serum

Serum was collected from blood which had been allowed to clot at 37°C for 30 minutes before being centrifuged at 2000g for 15 minutes at room temperature. Serum was removed and stored at -70°C in polypropylene tubes.

2.1.2 Platelet poor plasma

Platelet poor plasma was prepared by centrifugation of citrated (0.105M) blood samples at 2000g for 15 minutes twice. The top 2/3 of supernatant was removed after each step. Plasma was stored at -70°C in polypropylene tubes.

2.1.3 Buffy coat preparation

Buffy coats were prepared from whole blood taken into EDTA by centrifugation at 2000g for 15 minutes. The plasma supernatant was removed and the buffy coat carefully removed.

2.1.4 Pooled normal plasma

A pooled normal plasma (PNP) was prepared from samples of six volunteers. The PNP was determined to be negative for anticardiolipin LA and anti-β2GPI activity.

2.2 Tests for Antiphospholipid Antibodies

2.2.1 Anticardiolipin Antibody Assay

This assay involves coating a microtitre plate with cardiolipin then applying adult bovine serum (ABS) to the plate to act as a source of protein to bind to the negatively charged phospholipid and to block uncoated sites on the plastic plate. Following this dilute patient serum is applied and bound antibodies (directed against the protein/phospholipid complex) detected using immunoglobulin with anti-human antibody activity and alkaline phosphatase conjugate.

Method

- Rows A, B, E and F of a Nunc Polysorp® plate were coated with 30µl/well 50µg/ml cardiolipin (Sigma C-1649) in ethanol; the remaining rows were coated with ethanol only to serve as sample blanks.
- The plate was then incubated overnight at 4°C to allow the ethanol to evaporate.
- The plate was then washed three times with PBS and 75µl of (adult bovine serum) ABS (Sigma B-2271) diluted ten fold in PBS was added to each well. The plate was then incubated for 1 hour. This latter stage serves as both a blocking step in the protocol and provides a source of bovine proteins, which bind to the cardiolipin (including β2GPI).
- After washing three times with PBS, 50µl aliquots of doubling dilutions of reference sera (1/50 to 1/1600) and patient sera (1/50) were added to duplicate cardiolipin coated and blank wells. The standard serum was

obtained from a well-characterised patient with high titre IgG and IgM anticardiolipin antibodies with primary APS. The aCL titre of the standard was previously calibrated against a recognised standard reference serum. All sample and standard dilutions were made in 10% ABS in PBS.

- The plate was then incubated for three hours at room temperature and washed three times with PBS.
- 50µl of goat anti-human IgG (Sigma A-5403) or IgM (Sigma A-3275) diluted 1/1000 in 10%ABS in PBS were than added to each well and the plate was incubated for 90 minutes.
- After washing three times in PBS, secondary antibody binding was detected by the addition of 50µl/well of p-nitrophenyl phosphate disodium hexahydrate (Sigma 104-105) 1mg/ml in diethanolamine buffer (97g diethanolamine; 0.1g $MgCl.6H_2O$; 0.2g sodium azide in 1L water pH 9.8). The substrate reaction was incubated in the dark at 37°C for up to 45 minutes (until suitable colour had developed) and the reaction was stopped by the addition of 50µl 3M NaOH. The absorbance was then measured at 405nm using a plate reader.
- The mean OD of each blank well duplicate was then subtracted from the mean corresponding cardiolipin coated duplicate. Samples with a CV>10% were repeated. A standard curve was constructed on log/log paper for the standard samples and the patient samples aCL titre calculated from this in GPL and MPL units. The normal cut-off values in assay were 5 GPLU and 5 MPLU. (Defined by the 95^{th} percentile cut off

using data from 200 normal subjects for IgG and 50 subjects for IgM). The classification of patients with different aCL titres is shown in table 7.

Interpretation	GPL Units	MPL Units
Negative	<5	<5
Low Positive	5-20	5-20
Moderate Positive	21-79	16-59
High Positive	>80	>60

Table 7 Interpretation of anticardiolipin assay results

2.2.2 Anti β2GPI antibody assay

For this assay a commercial kit (Euro-Diagnostica) was combined with a standard (with known anti-β2GPI activity prepared in the haematology department at UCL). The assay employs a gamma-irradiated 96 well microtitre plate previously coated with human β2GPI, which is used to capture autoantibodies directed against the protein (if present) in a sample of sera from a patient. An enzyme conjugated monoclonal anti-human antibody to either IgG or IgM then detects the autoantibodies, and binding is quantified by means of reaction with substrate to the conjugated enzyme.

Method

- A standard curve was prepared by means of diluting the standard sera (of IgG or IgM) in the kit sample diluent from undiluted to 1/64 in doubling dilutions. The standard sera is from a well characterised patient with high aPL activity. This represents an activity range from 1.56-100% in the assay used.

- 100µl of test and standard samples diluted 1/100 in sample diluent were then added to wells in duplicate and incubated for 1 hour.
- The plate was then washed three times with the kit washing solution. 100µl of kit anti IgG or IgM conjugated antibody was them added to each well and incubated for 30 minutes.
- After washing, 100µl/well substrate (phenolphthalein monophosphate) was then added to each well and the reaction stopped once suitable colour development had taken place with the kit stop solution. The optical density of each well was then measured with a plate reader at 550nm. Samples with a CV>10% were repeated.
- A standard curve was prepared on log/log paper and test anti-β2GPI activity was calculated. Patients were considered positive for anti - β2GPI activity if they possessed a level $\geq$ 3.5% in the IgG assay and $\geq$ 3% in the IgM assay. Cut off values in the anti-β2GPI assay were defined as above the 95th percentile (80 individuals were used for the IgG cut off and 30 for the IgM cut off).

2.2.3 Tests for Lupus Anticoagulant (LA) activity

Dilute Russell's Viper Venom Time (DRVVT)

Principle

The principle of the test lies in the ability of aPL to interfere with the assembly of procoagulant proteins at negatively charged phospholipid surfaces and thus prolong the clotting time observed when RVV is added to plasma. When designing the test the concentration of phospholipid is adjusted to the

lowest concentration before its dilution becomes rate limiting. RVV activates factor X and will lead to a fibrin clot in the presence of V, prothrombin, phospholipid and Ca^{2+}. The DRVVT test is designed such that the dilution of the phospholipid and RVV make the test sensitive to interference by aPL with LA activity. Because RVV directly activates factor X, the test is not influenced by deficiencies in the contact system or of factors VIII, IX and XI. The test also employs a "platelet neutralisation step" in which the test is repeated in the presence of an increased phospholipid concentration, in this case using lyophilised washed normal human platelets, which bypass the LA activity. Because the detection of LA can be interfered with by the presence of activated blood cells and their fragments in the plasma being tested, it is essential that platelet poor plasma is used for testing. This is collected and prepared as described above.

Method

All LA testing was performed using a CA-1500 (Sysmex) automated coagulometer. Platelet poor plasma was prepared as outlined above. A commercially available LA kit the Unitest™ LA-DRVVT/PNR kit from Technoclone (Dorking UK) was used as per the instructions of the manufacturer. A DRVVT ratio of >1.2 with a failure to correct on 50:50 mix with normal plasma and correction by 10% or to within the normal range on testing with platelet neutralisation reagent were deemed positive for LA.

Taipan venom time

For patients receiving anticoagulant therapy with warfarin, the Taipan Venom time was used to detect LA activity. Taipan venom directly cleaves prothrombin to thrombin. As with the DRVVT, the reaction is phospholipid dependent and under the correct conditions sensitive to lupus anticoagulant activity. Tests were performed using platelet poor plasma on a CA-1500 (Sysmex) automated coagulometer. Taipan snake venom was obtained from Diagnostic Reagents (Oxford UK) and platelet neutralising reagent was the same as that used in the DRVVT test. A Taipan venom time ratio of greater than 1.1 with failure to correct on 50:50 mix with normal plasma and a correction of >10% with platelet reagent was deemed positive for LA activity.

2.3 Assay of binding of β2GPI to cardiolipin

An assay was developed for experiments examining the binding of proteolytically modified and polymorphic variants of β2GPI to negatively charged phospholipid surfaces. A version of this assay was published in a paper written during the period of this thesis (Nash *et al*, 2003).

- Nunc Polysorp plates were coated with cardiolipin overnight as per the anticardiolipin assay described in the section above. For this assay, the outer two rows of the plate (A and B; G and H) were blank wells and ethanol only was added to these wells overnight.
- The plates were then washed with 200μl PBS, then blocked for 1 hour with 1% Bovine Serum Albumin (BSA) (Sigma) and then washed again.
- The plate was then coated with a source of β2GPI 100μl/well; this was either plasma or a source of purified β2GPI (Scipac). The dilution of test samples varied between experiments and is given in the relevant sections. Samples were always loaded in duplicate for blank and cardiolipin coated wells, for some assays triplicate or quadruplicate wells were used. When plasma was used, the β2GPI concentrations for comparative samples were normalised, the concentration having previously been determined using the β2GPI antigen assay described later in this section.
- The plate was incubated for 90 minutes washed three times with PBS and then coated with a 1:500 dilution of Horse Radish Peroxidase (HRP) conjugated rabbit anti-β2GPI antibody (Dako PE854) in 1%BSA PBS to detect and quantify binding of β2GPI to the phospholipid surface, and incubated for 90 minutes.

- The plate was then washed again and conjugated antibody binding quantified by the addition of 100µl/well of tetramethylbenzidine dihydrochloride (TMB) (Sigma T3405) in phosphate citrate buffer with hydrogen peroxide (Sigma P-9305) made up according to the manufacturers instructions (1mg/10mls). The colour reaction was stopped at 3 minutes by the addition of 100µl/well H_2SO_4 (2M) and the optical density read at 450nm on an automated plate reader. Results were rejected and repeated where the CV for duplicate wells exceeded 10%. An example curve comparing the binding of β2GPI from pooled normal plasma and a patient with compound heterozygosity for the Cys306Gly and Trp316Ser β2GPI polymorphisms is shown in figure 10.

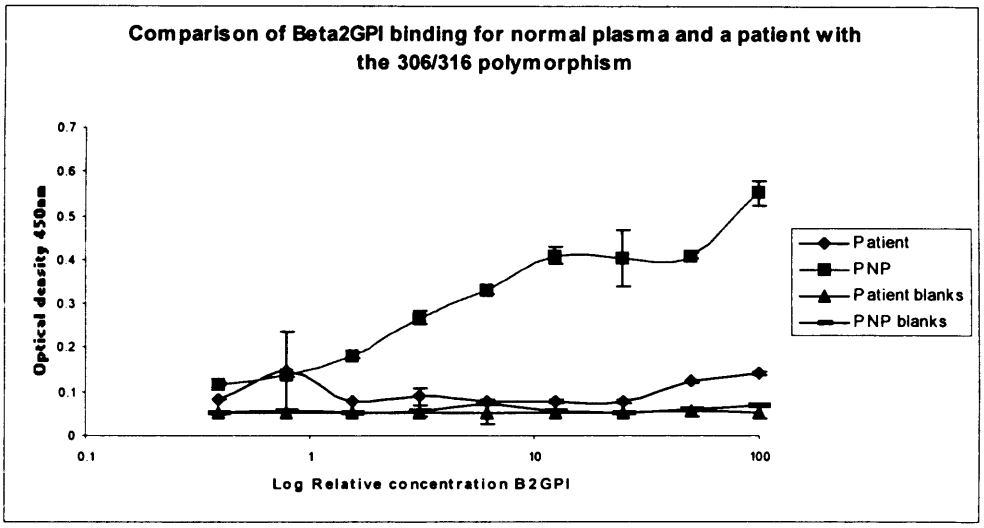


Figure 10 An example of a binding curve for normal plasma β2GPI and that from a patient who is a compound heterozygote for the 306 and 316 β2GPI polymorphisms.

Standard deviation bars are shown for duplicate wells, a relative concentration of 100 represents the concentration of β2GPI achieved by a 1 in 100 dilution of the normal plasma. (PNP=pooled normal plasma). Concentrations of β2GPI in the patient and PNP were normalised to each other by dilution in PBS.

2.4 β2GPI Antigen assay

For the purpose of normalising plasma concentrations of β2GPI to each other between samples and checking the concentration of β2GPI in samples during the work for this thesis, a method employing a sandwich ELISA was employed. The technique used was based on a method previously developed in our department (McNally *et al*, 1993).

- A Nunc Maxisorp plate was coated overnight with a 1 in 1,000 dilution of a rabbit anti-β2GPI IgG antibody (Dako 09152) (200μl/well).
- The plate was then washed three times with 300μl/well PBS; 0.1% Tween 20.
- A standard plasma with a known concentration of β2GPI (PNP 1/96 concentration β2GPI 223μg/ml) was prepared in doubling dilutions ranging from 1 in 3,000 to 1 in 96,000 in PBS Tween.
- Test samples were prepared in dilutions ranging from 1 in 6,000 to 1 in 24,000 in PBS Tween. Samples were loaded in duplicate for both test and standards and incubated for 2 hours.
- The plate was washed in PBS Tween followed by well loading with 100μl/well of 1/500 diluted HRP conjugated rabbit anti-β2GPI antibody (Dako PE854) in PBS and further incubation for two hours.
- The plate was washed again and conjugated antibody binding measured using TMB substrate and reading at 450nm as described in the β2GPI binding assay.
- A standard curve was constructed plotting optical density against log concentration using the standard plasma results. Sample concentrations

were then calculated using the results for each sample dilution that lay over the linear region of the standard curve. For standard and test samples, results were rejected where the CV for duplicate wells exceeded 10%.

2.5 Electrophoresis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) and western blotting

Method

SDS-PAGE with western blotting was used here primarily to detect the molecular weight change associated with plasmin (and kallikrein) cleavage of β2GPI. Prior to application to an electrophoresis gel, samples were diluted (in most cases either 1 in 4 or 1 in 10) in the following sample buffer containing DTT as a reducing agent: 62.5mM TRIS HCl; 2% (w/v) SDS; 10% (v/v glycerol; 0.01% Bromophenol blue; 360mM DTT; pH 6.8. Samples were placed in a boiling water bath at 100°C for three minutes before being centrifuged on a microfuge at full power (12,000g) for three minutes. A reducing buffer is necessary to detect the molecular weight change seen following plasmin action on domain of β2GPI since post cleavage, the molecule is still held together by a disulphide bond distal to the cleavage site.

For all the SDS-PAGE carried out in this thesis, BioRad® criterion gels were used, with the gel tank, running and transfer buffers as recommended by the manufacturer. Running buffer was made up as follows: 25mM TRIS; glycine 192mM; SDS 0.1%; pH8.3. 10% (BioRad® 345-0009) and 8-16% gradient (BioRad® 345-0037) gels were used. 45μl of sample in reducing buffer was loaded into each well of the gel. A biotinylated standard was used to allow molecular weight determination on the western blot (BioRad® 161-0319) with a molecular weight range from 200,000 to 6,500 Daltons. Electrophoresis was carried out at 200 volts for 55 minutes. The gel was then blotted onto

nitrocellulose using a semi dry transfer system with the following transfer buffer: TRIS 25mM; glycine 192mM; methanol 20% (v/v); pH 8.3. Gel to nitrocellulose transfer took place at 0.4 amps for 30 minutes. The nitrocellulose blot was blocked for 1 hour in 5% powdered milk (Marvel®) in borate buffered saline - Tween (BBS-T): H_3BO_3 (0.02M), NaCl (0.15M), Tween-20 (0.1%), pH 7.2. The nitrocellulose was then washed three times in BBS-T and incubated overnight in BBS-T containing a 1 in 1,000 dilution of Rabbit anti-human β2GPI polyclonal IgG (Dako 09152). The nitrocellulose was then washed three times in BBS-T and incubated for two hours with a 1 in 1,000 dilution of Swine anti-Rabbit immunoglobulin with Alkaline Phosphatase conjugate and a 1 in 2,500 dilution of Alkaline Phosphatase conjugated avidin (BioRad® 170-6533) (to detect the biotinylated molecular weight standards) in BBS-T. The nitrocellulose then was washed 3 times in BBS-T and developing solution was then added (ethanolamine buffer, (0.1M) (50ml) pH 9.6 containing: nitro blue tetrazolium (5 mg), methanol, (500µl) acetone (250µl), 5-bromo-4-chloro-3-indolyl phosphate (3mg) and $MgCl_2$ (4mM)). The paper was left to develop for a few minutes until staining was of sufficient intensity before finally washing with H_2O. An example western blot is shown in figure 11.

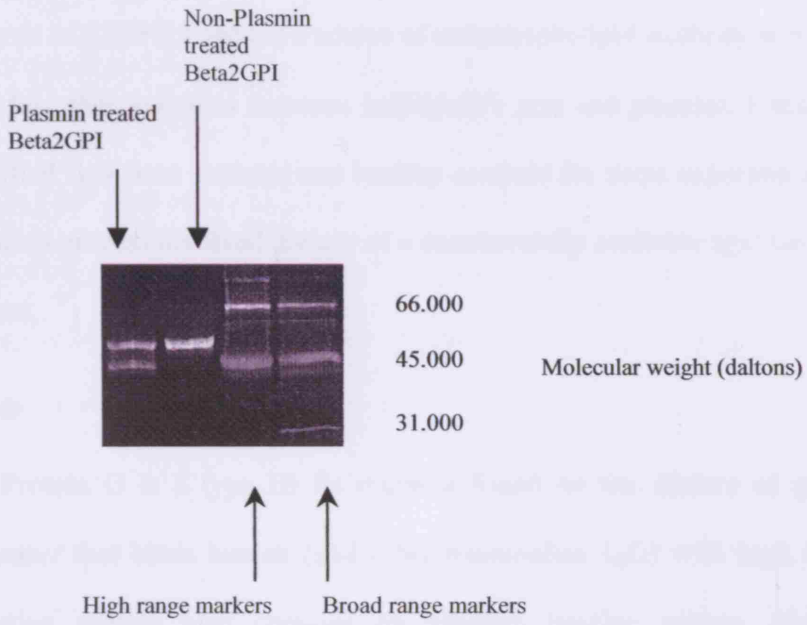


Figure 11 Western blot on cellulose acetate of β2GPI treated with plasmin and control.

Samples were treated as outlined in the methods in reducing buffer and ran on an 8-16% gel. Here a second molecular weight standard (BioRad® 161-0311) has also being run to confirm the position of the 45,000 Dalton marker (the lowest marker in this standard).

2.6 Preparation of Immunoglobulin G fractions

To carry out the experiments described in this thesis investigating the effect of the presence of aPL on fibrinolytic processes (at cell surfaces and the proteolysis of β2GPI) I needed a source of antiphospholipid antibody activity. To control for other variables between individual's sera and plasmas, I decided to use purified IgG from patients and healthy controls for these experiments. This purification process involved the use of a commercially available agarose-protein G column.

Principle

Protein G is a type III Fc receptor found on the surface of group G *streptococci* that binds human (and other mammalian IgG) with high affinity. The native protein also contains an albumin binding region. Amersham Pharmacia produce a recombinant protein G with two IgG binding sites and with the albumin binding site deleted. The protein G is then coupled to highly cross linked agarose beads by N-hydroxysuccinamide activation, the matrix bound protein G is then packed into a column for use. The protein G columns used in this work were HiTrap Protein G 5ml columns (Amersham Pharmacia 17-1128-01).

Method

Sera from pooled normal or patient samples were diluted 1 in 10 in PBS (pH 7.2) and passed slowly over the protein G column using a peristaltic pump system. The protein G column was washed with PBS until the absorbance of the flow through at 280nm (measured using a spectrophotometer with comparison made to an appropriate buffer containing blank cuvette) was back to a base line

previously established for the column. (Proteins absorb light at 280nm in proportion to their content of aromatic amino acid residues). The IgG isolated on the column was then eluted using 0.1M glycine pH 2.8, fractions were collected in 4ml aliquots into 1M TRIS pH 9 to neutralise the acid pH. For a 5ml serum sample diluted in 50 mls PBS the first seven aliquots of eluate were found to contain the bulk of the IgG isolated. The elution curve was measured by monitoring the absorbance at 280nm. After each sample was eluted the column was "washed" in glycine until the absorbance at 280nm had fallen to baseline and then washed in PBS to remove the glycine prior to next usage.

For use in my experiments, the IgG in TRIS/glycine was exchanged into a commercially available PBS (Dulbeco's PBS Invitrogen Ltd). This was carried out using an Amicon concentrator system under pressure from compressed nitrogen over a membrane with a 30,000 Dalton cut off (Amicon PM30). The collected fractions underwent six 3x volume exchanges (equivalent to 3x3x3x3x3x3=729 times dilution of original buffer) using this ultrafiltration system. This reduced the glycine content of the buffer to 0.14mM. When diluted later in experiments with other components, this meant the glycine content of buffer was roughly that found physiologically.

The concentration of IgG in the final solution was calculated using Beer's Law after measuring the absorbance at 280nm using an extinction coefficient of $E^{1\%,\ 1cm}$=13.6. The absorbance at 260nm was compared to that at 280nm to ensure the ratio of the two was less than 0.6. IgG fractions were stored prior to use at -20°C. Prior to use in experiments the IgG fractions were filtered over a 200nm cut off filter and the protein concentration re-calculated by measuring absorbance at 280nm and Beer's Law.

2.7 Culture of vascular endothelial cells

Background

The *in vitro* culture of vascular endothelial cells has been possible since the development of cell culture techniques in the 1970's. Human umbilical vein endothelial cells (HUVEC) can be obtained via the collagenase digestion of the interior of the umbilical vein followed by cell culture in flat bottomed flasks containing nutrient rich media (Jaffe *et al*, 1973).

Reagents, cell culture media and passage

- HUVEC were obtained from TCS cellworks in cryopreserved state (ZHC2101 pooled donor).
- They were grown to confluence in $80cm^2$ flat-bottomed cell culture flasks until confluent. Flasks were pre coated with 1% gelatin (Sigma G1393) prior to use. ZHM2953 Large vessel Endothelial Growth Medium Package (TCS cellworks) (with serum supplement and gentamycin/amphoterecin added as per the manufacturer's instructions) was used as growth media.
- Cells were incubated at 37°C with 5% CO_2.
- For all experimental work HUVEC of less than passage six were used.
- At confluence cells were washed with Hank's balanced salt solution (Invitrogen), Accutase cell detachment media (TCS cellworks) 5ml was added and the cells incubated for 2 minutes. The flask was then tapped gently to release the cells from the flask base and 10ml of growth media added. The cells were then transferred to a sterile tube and centrifuged at

200g for 10 minutes. The supernatant fluid was then removed and the cells resuspended in 20mls culture media.

- For the purpose of passage, 3mls of resuspended cells were added to 12mls of media and added to another gelatin coated 80cm^2 culture flask.
- For the purpose of cell culture on 96 well plates (see chromogenic assay and florescent plate reader experiments), 200µl of the resuspended cell culture fluid was added to each well of a previously gelatin coated Costar 3595 (for chromogenic assays) or a Povair 215006 (for fluorescent assays) plate. For "blank" wells culture media was added which contained no cells.

2.8 Fluorescent plate reader studies

The experiments performed on HUVEC monolayers measuring the binding of FITC labelled plasminogen to the cell surface were performed using a BMG Labtech FLUOstar optima fluorescent plate reader. For the measurement of FITC conjugated protein, an excitation wavelength of 485nm and emission wavelength of 520nm was employed. Data acquisition was performed via the FLUOstar optima software provided by the plate reader manufacturer.

2.9 Flow cytometry

Some provisional work on the binding of plasminogen to HUVEC in this thesis was performed using flow cytometry. This technique allows measurements to be made on individual cells as they flow in a fluid stream through a sensor. The flow cytometer measures the scatter of light from cells as they pass through a laser. This scatter is proportional to the size (forward scatter) and granularity

(side scatter) of the cell. Moreover, fluorescent dyes bound to the cell surface (conjugated to protein or antibody) fluoresce when light of the correct wavelength is applied, this fluorescence can be quantified by means of a photomultiplier tube.

For all flow cytometry experiments in this thesis, a Coulter Epics XL-MCL flow cytometer was used. For all experiments, a previously established gating strategy for HUVEC based on S-ENDO 1 expression was employed and at least 10,000 events within the endothelial cell gate recorded. Details of individual experiments are given in the relevant results section. A typical scatter plot for HUVEC and an illustration of basal S-ENDO 1 (CD146) is shown in figure 12.

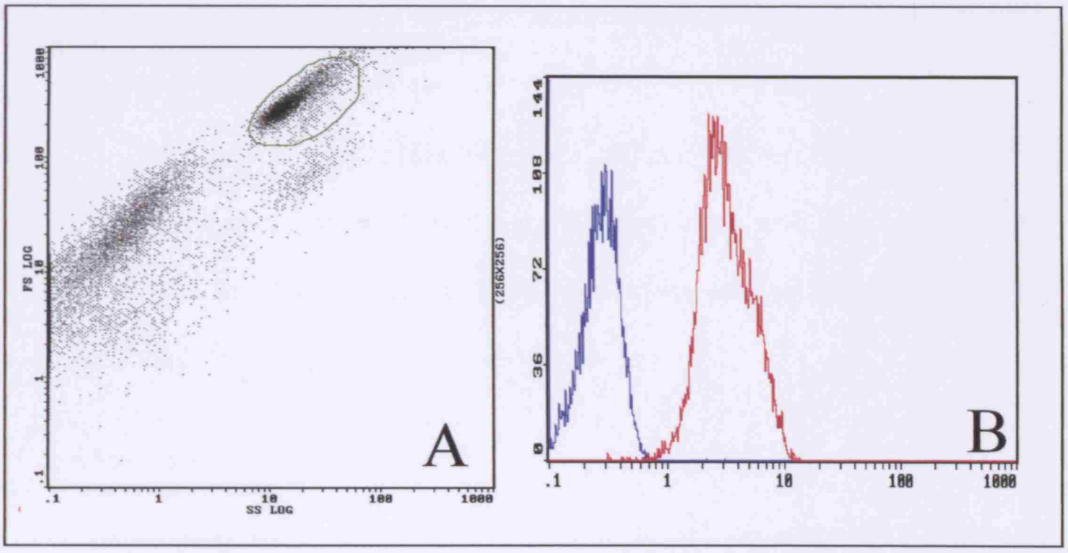


Figure 12 HUVEC scatter plot (A) and CD146 positivity (B)

In (B) binding of anti-CD146 antibody (Biocytex) is shown in red compared to isotype control at the same concentration (mouse IgG1) in blue. >99% of the cells gated in (A) were CD146 positive. The HUVEC gate is shown in green in (A). Test and control antibodies were used as per the manufacturer's instructions.

2.10 Preparation of Paraformaldehyde fixative

A 25% (w/v) stock solution of paraformaldehyde (PFA) was produced as per the method of Hannah *et al* (Hannah *et al*, 1998). PFA was weighed in a beaker and 10% of the final volume required of 10x concentrated PBS was added. Water was added up to 90% of the volume required and the beaker placed in a water bath at 65°C. Once the solution reached 65°C, 10M NaOH was added in drops until the solution cleared. The solution was allowed to cool and made up to its total volume. The stock solution was aliquoted and stored at -20°C. For use at 1% strength the 24% solution was diluted when needed to 1% strength in PBS.

Chapter 3

Analysis of the antiphospholipid antibody positive patient cohort at University College London Hospital

3.1 Introduction

In order to carry out the experimental work performed for this thesis, it was necessary to examine the characteristics of the large number of patients seen at our centre with positivity for aPL. This examination was with particular reference to the level and persistence of positivity for aPL, which would identify subjects likely to be useful to study. As discussed later, this work was expanded in scope to look at a new serological classification for APS which was being discussed at the time of this work.

This chapter will describe the patient cohort at our centre and also present background data on the patients the purified IgG from who was used in later experiments. Some of this work in this chapter is presented in the literature (Nash *et al*, 2004).

3.1.1 Aims of this section

1) To examine the patterns of seropositivity for aPL in our patient cohort at UCLH with a view to identifying patients for study in later experimental work.

2) To use the data in 1) to test a newly proposed serological classification of the antiphospholipid syndrome.

3.2 Analysis of the pattern of aPL serology in a cohort of patients at UCLH over the period 2000-2003

3.2.1 Background

During the course of my work, the scientific and standardisation subcommittee (SSC) of the International Society for Thrombosis and Haemostasis (ISTH), at their meeting in Boston (July 2002) discussed a reclassification of APS based on a new serological classification (Chairman J Arnout, 2002). This new classification (table 8) suggested the replacement of the aCL test with tests more "direct" for anti-β2GPI activity detection. The minutes of this meeting made a specific request for investigators to "try-out" this new classification on their cohorts. The work I had already performed in analysing our cohort of patients placed me in a position to "field test" this new classification with a large cohort of APS patients. To fully classify the patients some retrospective analysis of previously banked plasma and serum samples was necessary.

Type I:	Anti-β2GPI antibody and LA positive
Type II:	LA positive only
Type III:	Anti-β2GPI antibody positive only
Type IV:	"All the rest" (anti-phosphatidyl ethanolamine, anti-prothrombin etc)

(Each test positive on two occasions at least six weeks apart)

Table 8 The serological classification of APS proposed by the Boston 2002 SSC meeting

Arguments for this form of classification for APS date back to the discovery in the early 1990's that the antibody interaction with cardiolipin in the

aCL assay is dependent on binding to proteins which interact with negatively charged phospholipid, the first of which to be discovered was β2GPI (Galli *et al*, 1990) (McNeil *et al*, 1990). The sera of patients with APS may also contain antibody activity directed against other proteins which interact with negatively charged phospholipid surfaces such as annexin V (Arnold *et al*, 2001) and prothrombin (Galli & Barbui, 1999). The clinical relevance of some of these other antibody activities is still to be established. Recent meta-analyses have revealed LA positivity to be the strongest risk factor for thrombosis in APS patients. The odds ratio for thrombosis in persistently positive LA patients being 5-15 times higher than controls (Galli *et al*, 2003b). The same group has also recently carried out a meta-analyses on anti-β2GPI and anti-prothrombin antibody positivity (Galli *et al*, 2003a), this work concluded that more work was needed to establish these activities as independent risk factors for thrombosis.

3.2.2 Methods

I collated data on 123 consecutive patients attending the haematology outpatient department at University College London Hospitals (UCLH) NHS Trust manifesting persistent aPL positivity over the period 2000-2003. The patient sera and platelet poor plasmas were tested for aCL and anti-β2GPI activity of IgG and IgM class and for LA as described in the general methods section and outlined here.

Anti-β2GPI antibodies were measured using a commercial kit (see general methods section), based on a method developed in our Department (McNally *et al*, 1995). An in-house standard (comprising an index human serum

from a well-characterised patient with APS and a known high concentration of anti-β2GPI antibodies) was used to construct the calibration curve in both IgG and IgM assays. Patients were considered positive for anti-β2GPI activity if they possessed a level ≥ 3.5% in the IgG assay and ≥ 3% in the IgM assay. Cut off values in the anti-β2GPI assay were defined as above the 95th percentile (80 individuals were used for the IgG cut off and 30 for the IgM cut off).

Anticardiolipin assays were performed using an in-house assay (McNally *et al*, 1995) based on the work of Loizou *et al* (Loizou *et al*, 1985), standardised using international reference materials (Harris *et al*, 1987;Loizou *et al*, 1985). The normal cut-off values in the aCL assays were 5 GPLU and 5 MPLU. (Defined by the 95th percentile for 200 normal subjects for IgG and 50 subjects for IgM).

After screening APTT and appropriate mixing studies (50:50 mix with normal plasma), LA activity was detected using a commercial kit for the dilute Russell's viper venom time (DRVVT) employing a platelet neutralisation procedure (see general methods section). LA activity was present if the DRVVT ratio with dilute phospholipid was ≥ 1.2 and the time corrected by ≥ 10% or to within the normal range in the platelet neutralisation procedure. The taipan venom time method was also used to confirm LA in patients on warfarin (Rooney *et al*, 1994). Testing complied with the recommendations of the Haemostasis and Thrombosis Task Force of the British Committee for Standards in Haematology (Greaves *et al*, 2000).

For the most part these tests were performed in the routine lab at UCLH NHS Trust. However, for some patients retrospective testing of previously banked specimens of serum was necessary either to confirm persistent antibody

positivity over time or to check for activity which has not been assayed as part of routine work up (in most cases this was IgM anti-β2GPI activity which was not made part of the routine APS "work-up" until 2002).

Patients were only considered positive for an aPL test if they manifested positivity on two occasions at least six weeks apart. Data on the presenting complaint of the patients were also collated.

The pattern of aPL positivity seen in the patients was analysed using a system based on the proposed classification of the ISTH SSC meeting of 2002 (Chairman J Arnout, 2002). In our analysis we took the liberty of changing the type IV "all the rest" group to be that with aCL positivity only. Patients were split into groups with LA and anti - β2GPI positivity (type 1); with LA activity alone (type 2); with anti - β2GPI positivity alone (type 3) and with aCL positivity alone (type 4). Patients were allocated to the first three groups irrespective of their aCL results. The mean titre of aCL and anti - β2GPI antibodies of each patient was calculated. The median titres of each subgroup were compared using the Mann Whitney U test (a p value of <0.05 was taken to represent statistical significance).

3.2.3 Results

One hundred and twenty-three patients with persistent aPL were included in the analysis. The clinical presentations in the cohort are summarised in Table 9. Of these patients, 96 had clinical features consistent with the Sapporo criteria. Seventy of the patients had presented with proven arterial and/or venous thrombosis. The distribution of the 96 patients amongst different subgroups is shown in figure 13.

The median aCL IgG level in patients who were also positive for both LA and anti - β2GPI antibodies was significantly higher than that seen in the other groups (Table 10). This finding was consistent when data from patients with thrombotic complications were analysed (Table 11). Thirty-four out of 96 of the patients in the Sapporo clinical criteria group were negative for IgG aCL and 36 were negative for IgM aCL. Twelve of the patients in this group were positive for both IgG and IgM anti - ß2GPI.

CLINICAL PRESENTATION	NUMBER
Arterial Thrombosis*	34
Venous thrombosis*	29
Obstetric complications*	25
Family History of Thrombosis	8
Miscellaneous	7
Thrombocytopenia	6
Arterial Thrombosis and Obstetric complications*	3
Migraine	3
Arterial and Venous Thrombosis*	2
SLE	2
Arterial Thrombosis and SLE*	1
Obstetric complications and SLE*	1
TTP	1
Venous thrombosis and SLE*	1
Total	123

Table 9 Distribution of clinical presentations in the aPL positive cohort studied

* = Sapporo clinical criteria positive

Subgroup	IgG aCL (GPLU)	IgG anti - B2GPI %	IgM aCL (MPLU)	IgM anti - B2GPI %
LA and anti - B2GPI antibody positive[+]	*57 (0.5-107)	**34 (0.9-206)	***4.8 (0-85)	11.5 (3-52)
LA positive[+]	1.7 (0.2-50)		0.5 (0-35)	
anti – B2GPI positive[+]	6.65 (0-31)	3.75 (0.8-142)	0.4 (0-50)	5.2 (1-144)
aCL positive only	7.1 (0.1-55)		1 (0-13)	

Table 10 Median (and range) aCL and anti anti-ß2GPI titres for patients positive for Sapporo clinical criteria (n=96)

[+] +/- aCL positive only

* significantly different to other groups (p=0.001 (LA positive), p=0.0003 (anti ß2GPI positive) and p=0.0014 (aCL only) using Mann Whitney test)

** significantly different to anti ß2GPI positive group (p=0.0012 Mann Whitney test)

*** significantly different to other groups (p=0.04 (LA positive), p=0.004 (anti ß2GPI positive) and p=0.01 (aCL only) using Mann Whitney test)

Subgroup	IgG aCL (GPLU)	IgG anti - B2GPI %	IgM aCL (MPLU)	IgM anti - B2GPI %
LA and anti - B2GPI antibody positive[+]	*57 (0.5-107)	**34 (1-206)	4.8 (0-85)	11.5 (3-52)
LA positive[+]	1.2 (0.2-34)		1.4 (0-35)	
anti - B2GPI positive[+]	4.9 (0-31)	4 (0.8-18)	0.3 (0-50)	5 (1-88)
aCL positive only	5.9 (0.1-48)		3.7 (0-13)	

Table 11 Median (and range) aCL and anti anti-ß2GPI titres for patients with arterial and/or venous thrombosis (n=70)

[+] +/- aCL positive only

* significantly different to other groups (p=0.0009 (LA positive), p=0.0007 (anti-ß2GPI positive) and p=0.001 (aCL only) using Mann Whitney test)

** significantly different to anti ß2GPI positive group (p=0.0014 Mann Whitney test)

25.2% (31/123) of all the patients studied and 26% (25/96) of those positive for Sapporo clinical criteria had aCL (IgG and/or IgM) positivity alone and were negative for LA and anti - β2GPI antibodies on repeat testing. The clinical presentations of the patients in this group are shown in Tables 12 and 13. Seventeen of the patients in this group had suffered a thrombotic event. The

group of patients with aCL positivity alone had aCL levels which were significantly lower than the group with both LA and anti - β2GPI antibodies, their aCL levels however were not significantly different to those with LA or anti - β2GPI antibodies alone (Tables 10 and 11). The mean aCL IgG level in the Sapporo clinical criteria positive group with aCL only was 11.5 GPLU. The mean IgG aCL level in patients with arterial thrombotic events and aCL only was 10.4 GPLU.

The mean individual levels of aCL and anti-β2GPI antibodies seen in patients in the different serological groups are shown in figures 14 and 15. Patients with IgG aCL levels in excess of 60 GPLU were in all cases found to be positive for LA and anti - β2GPI antibodies.

Diagnosis	Number
Ischaemic stroke	12
Venous Thrombosis	5
Late Pregnancy Morbidity	2
Recurrent Miscarriage	6

Table 12 Clinical presentations of patients positive for aCL but negative for LA and anti-β2GPI antibodies with Sapporo clinical criteria

Diagnosis	Number
Thrombocytopenia	1
Thrombotic Thrombocytopenic Purpura	1
Migraine	1
Familial APS (no symptoms)	2
Miscellaneous	1

Table 13 Clinical presentations of patients positive for aCL but negative for LA and anti-β2GPI antibodies without Sapporo clinical criteria

Patient	aCL IgG	aCL IgM	B2 IgG	B2 IgM
1	21.6	0.7	1.5	1.6
2	14	0	0.8	3.3
3	8.3	0.3	0.9	2.6
4	5.9	0	1.1	1.5
5	2.4	4.5	1.3	1.7
6	6.1	5.4	2	1.2
7	1.8	4.2	0.8	1.5
8	5.6	3.7	1.2	4.4
9	9.9	0.4	0.9	2.4
10	0.1	4.7	0.7	1.9
11	48.7	1.1	0.4	2.7
12	0.4	7.9	0.8	2.2
13	0.3	6.5	0.6	1.3
14	0.3	8.6	0.9	3.3
15	1.9	13.2	1.8	1.7
16	12.4	0.1	0.2	1.2
17	7.1	0.1	0.9	1.7
18	5.1	2.3	2.4	3.6
19	7.5	0.04	0.6	2.5
20	19.6	0.08	0.7	3
21	6.4	0.9	0.7	3.2
22	8.2	0.2	2.2	1.8
23	55	0.1	0.5	2.7
24	20.4	0.15	0.6	1.1
25	19.1	1.05	0.85	2.5

Table 14 mean aCL and anti-β2GPI titres for patients with Sapporo clinical criteria who were persistently aCL positive only (all patients were persistently LA negative)

Full thrombophilia screening data was available in full for 18 of these patients. Two of the patients were heterozygous for the prothrombin G20210A mutation (17 and 25).

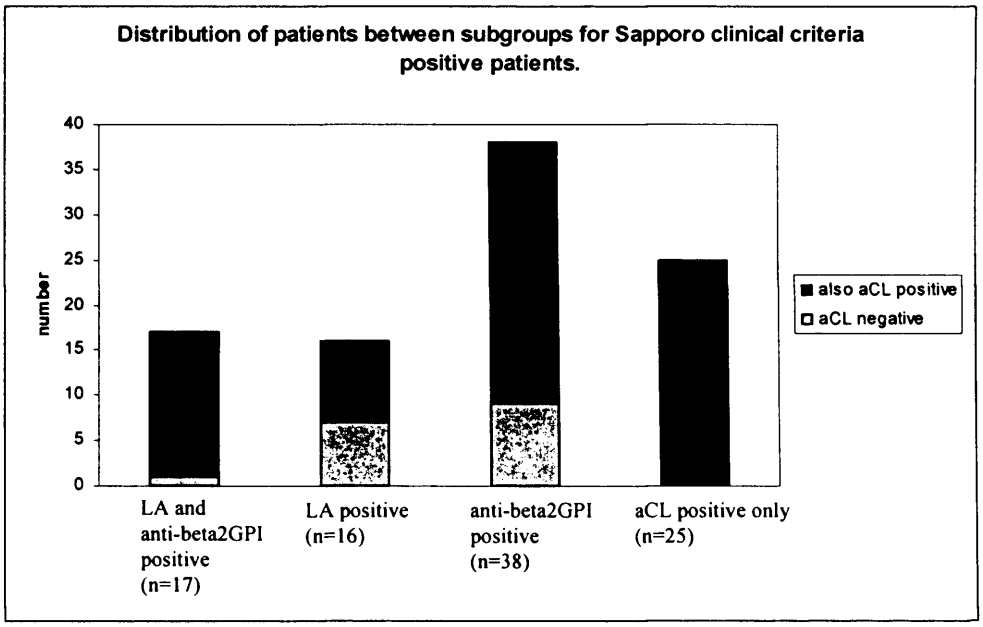
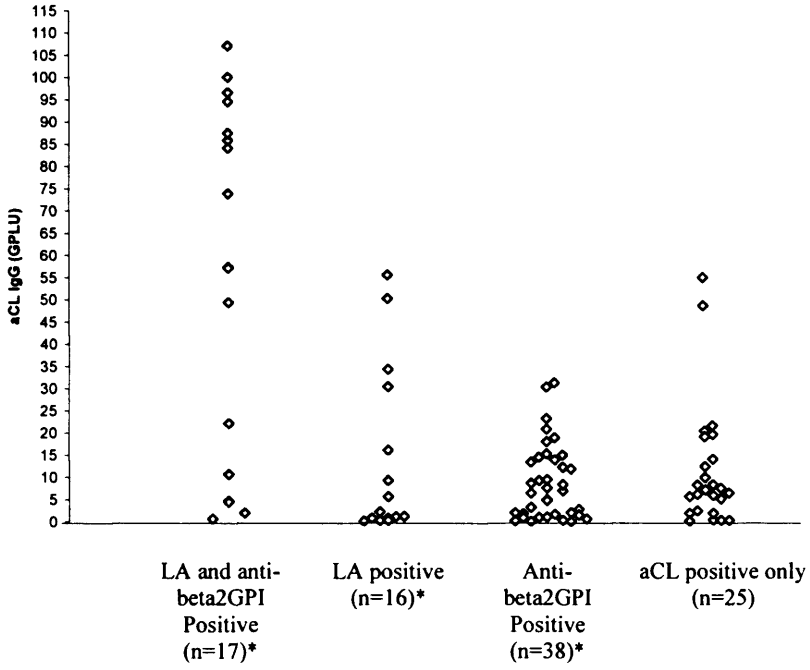


Figure 13 Distribution of patients positive for Sapporo clinical criteria between subgroups (aCL positive = IgG and/or IgM)

(The numbers of patients negative for both IgG and IgM aCL in the first three groups were 1, 7 and 9 respectively).

(a)



(b)

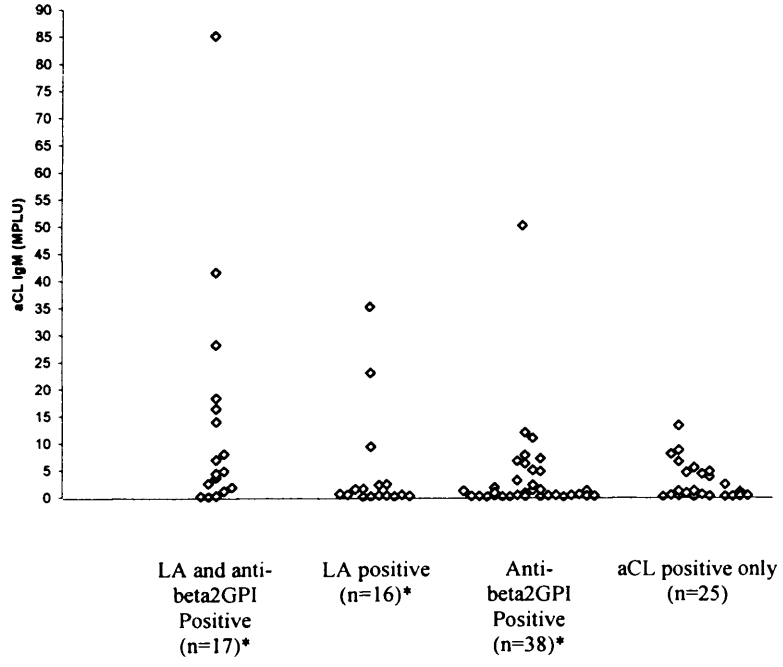
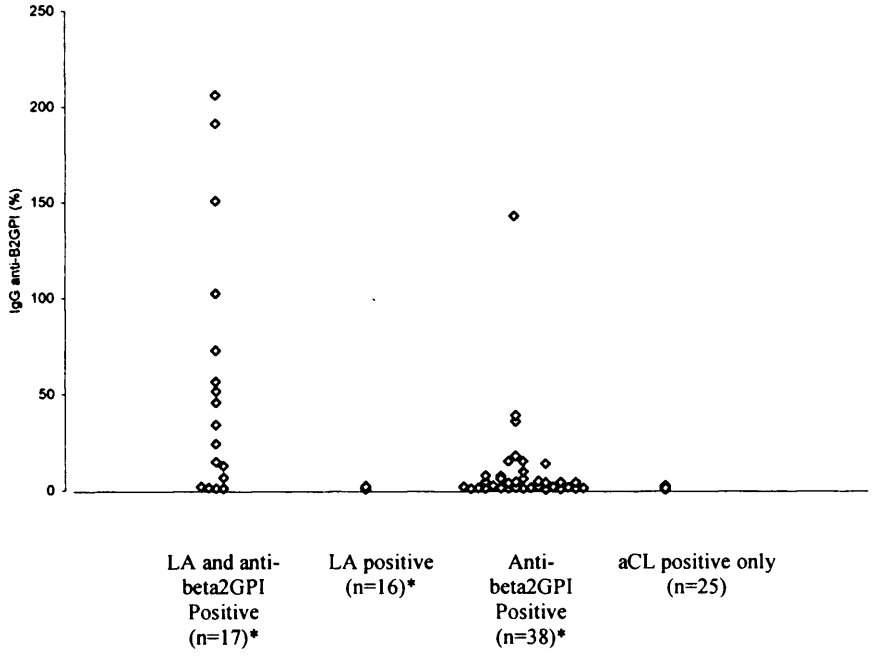


Figure 14 Scatterplots illustrating the mean aCL IgG (a) and IgM (b) levels for Sapporo clinical criteria positive patients in each of the serological groups examined.

* - may also be aCL IgG or IgM positive

(a)



(b)

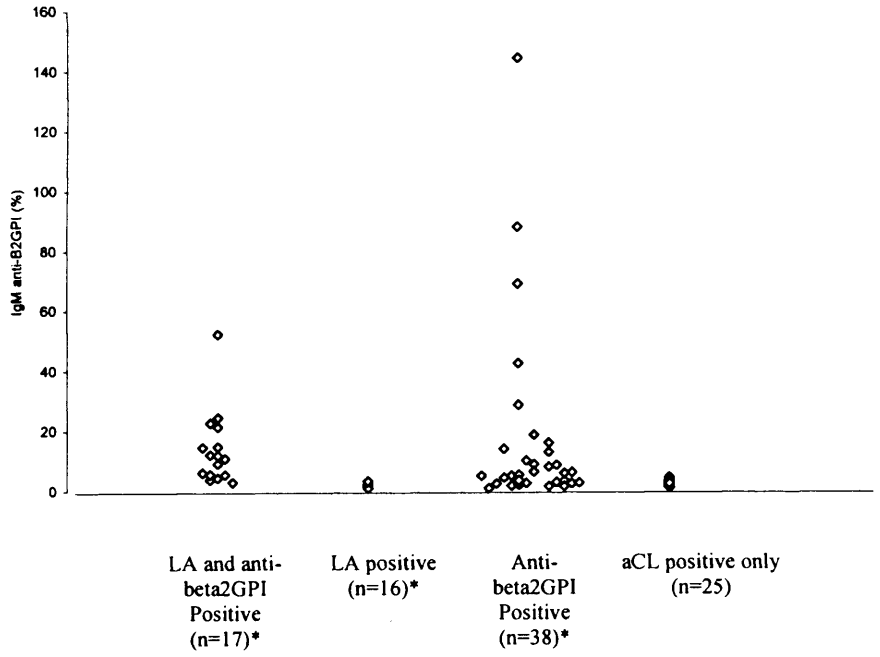


Figure 15 Scatterplots illustrating the mean IgG (a) and IgM (b) anti-β2GPI levels for Sapporo clinical criteria positive patients in each of the serological groups examined

* - may also be aCL IgG or IgM positive. Note by definition, patients in the 2nd and 4th groups were anti-β2GPI antibody negative.

3.2.4 Discussion

More than one quarter of the patients in this cohort with Sapporo clinical criteria for APS were found to be positive for aCL but negative for LA and anti - β2GPI antibodies. This data would seem to indicate that omission of aCL testing in favour of only testing for LA and anti - β2GPI antibodies could result in a failure to diagnose APS in some patients. This could lead to significant changes in patient management. In particular, these management changes would include decisions regarding initiation, intensity and duration of anticoagulant and anti-platelet therapy and the use of aspirin and heparin during the antenatal period. A similar proportion of aCL positive but LA and anti-β2GPI negative patients has been reported in another recent study (Alessandri *et al*, 2005). In our cohort, the presence of a persistent IgG aCL level of more than 60 GPLU is strongly predictive of the presence of co-existing LA and anti - β2GPI antibodies.

Twenty-seven patients in this study manifest persistent aPL but do not fit the criteria for a diagnosis of APS based on the Sapporo clinical criteria. Of particular note are the number of patients with thrombocytopenia. An association between aPL and peripheral consumptive immune thrombocytopenia has been described (Galli *et al*, 1996). In some APS patients with thrombocytopenia, antibody activity directed against platelet glycoproteins (IIb-IIIa and Ib-IX) have been demonstrated (Macchi *et al*, 1997).

Our data suggest that the laboratory diagnosis of APS should continue to include testing for LA, IgG and IgM anti - β2GPI antibodies and aCL, since this maximises the chance of detection. A recent multicentre study has shown that the aCL ELISA is the most common immunoassay used in the routine detection of aPL in the laboratories questioned. The same study however has highlighted the

sometimes poor agreement between laboratories with regard to aCL results. These differences could however be mitigated by the adoption of consensus protocols and calibration standards between centres. An aim for the future should be international standardisation of the test to allow greater comparison of results between centres (Tincani *et al*, 2001).

The patients with aCL who are negative for anti - β2GPI antibodies are likely either to have aPL which are directed against another protein/phospholipid complex e.g. prothrombin, or have antibodies which are unable to recognise β2GPI in the conformation it assumes when directly bound to a "high-binding" ELISA plate. There is also some evidence that in some purification processes, β2GPI may be cleaved by proteolysis. This may mean that some commercial anti-β2GPI kits are coated with an antigenically distinct form of the protein (personal communication, P de Groot). In either case, the aCL assay retains its importance in the diagnosis of APS and remains much more practical in the busy hospital laboratory setting than "extended" assays for antibody activity against other proteins, many of which have undetermined clinical application.

A criticism of my conclusion with regard to the use of the anticardiolipin assay in this cohort could relate to the observation that many of the patients in the "aCL only" group had low titres for aCL. While this is indeed true, four of the patients had mean IgG aCL titres in excess of 20 GPLU and eight greater than 10 GPLU. A clinical example of a patient with positivity for aCL only in which significant management changes in anticoagulation treatment were made (partly) on the basis of persistent aPL positivity is outlined in table 15.

Patient 1		
Female	Age 60y	
Presentation:	Recurrent cerebral infarction and livedo reticularis (Sneddon's Syndrome)	
aPL titres:	IgG aCL 21.6 GPLU	IgM aCL 0.7 MPLU
	LA negative	
	IgG anti-β2GPI 1.5%	IgM anti-β2GPI 1.6%
	(mean titres over 6 separate tests over a 3 year period)	
	Normal protein C, protein S, antithrombin, APCR, Prothrombin G20210A mutation not detected, heterozygous for thermolabile MTHFR variant	
Management:	Anticoagulation with warfarin (target INR 3) and Aspirin 75mg po od	
	Severe CVA while on anticoagulation (INR=2.7)	

Table 15 Case summary of one of the patients in the "type IV" cohort

Some further examples of patients with persistent positivity for aCL only emerged during the time following the cohort analysis presented above. These patients have not been added to the analysis of the large cohort since this was performed on consecutive patients presenting to our clinic and adding these patients into the data would skew in favour of the type IV group. The details of these additional "type 4" patients are shown in table 16.

Patient	Clinical presentation	Mean IgG aCL	Mean IgM aCL	Mean IgG anti Beta2GPI	Mean IgM anti Beta2GPI
1	Multiple PE	16.8	0	0.6	1.5
2	ITP	5.9	0	1	2.2
3	DVT	10.8	1.3	0.9	1
4	Recurrent Miscarriage	12	0.5	0.6	1.5
5	Recurrent Miscarriage	6.1	0.2	1	2.2
6	ITP	26	0.4	0.7	1.7

Table 16 Characteristics of six patients found to be persistently positive for aCL only

By definition all of these patients were persistently negative for LA. All patients were sampled on two occasions at least six weeks apart. ACL IgG and IgM results are expressed as GPLU and MPLU respectively. One of the patients is a V Leiden heterozygote (3) and one has treated polycythaemia (4).

Since this study was performed, a revised consensus statement on the classification of APS has been published (Miyakis *et al*, 2006). Importantly this new paper suggests that testing for repeat aPL positivity is performed at least 12

weeks apart and that anti-β2GPI positivity is included in the definition of the syndrome. This new classification also suggests that for a diagnosis of definite APS a cut off of 40 GPLU or MPLU is employed in the aCL test. With regard to our cohort at UCLH, this cut off would clearly result in many of our patients not fulfilling this criteria, including some of those with repeat LA and anti-β2GPI positivity. Part of this relates to the suggestion of the use of a 99th centile cut off for aCL positivity in the new guidelines, while our group is still employing a 95th centile cut-off. Whether in our clinical practice at UCLH this new cut off should be addressed is beyond the scope of discussion of this thesis. However the cut offs used at UCLH have clinical utility in our hands. Moreover, given the heterogeneity of results associated with aCL testing perhaps further work could involve examining our lower titre samples at another centre.

The referral pattern of patients to our centre at UCLH is likely to be different to that of other centres. Our unit has fewer patients with connective tissue disease and autoimmune problems than other centres because these patients are seen directly by the rheumatology team. In addition our centre has a relatively large obstetric practice compared to other units. These factors also need to be borne in mind when considering the serological patterns seen in this study.

My final word on the patterns of serological positivity seen in our cohort would be to emphasise that this work indicates the importance of performing different serological tests to diagnose APS, ideally aCL, LA and anti-β2GPI. The importance of looking at the pattern of positivity across all three tests was illustrated in a recent study (Pengo *et al*, 2005). This study highlighted the increased risk for thrombosis in patients with "triple positivity" for aPL as

compared to those with double positivity but negative for LA. All three tests for aPL should be employed together when possible.

3.3 A summary of the patients whose samples were used in the subsequent work in this thesis.

The experimental work in the remainder of this thesis (with the exception of the work on the β2GPI 306 polymorphism) using patient samples positive for aPL, concentrated on a few patients with aPL positivity from our cohort. In the main these patients were chosen because they exhibited high titre aPL which would be suitable for purification of the IgG fraction (most were also LA positive). All the patients whose samples were used were formally consented. A summary of the patients from whom aPL were purified for later use is shown in figure 17. Some of the patients listed here presented after the work on the large cohort was performed and are not part of that data set.

Patient	Clinical presentation	aCL IgG	aCL IgM	LA	anti B2GPI IgG%	anti B2GPI IgM%
1	Multiple VTE	84	42	pos	191	52
2	VTE, Lower limb ischaemia	100	7	pos	115	15
3	Familial APS, Cognitive problems	53	17	neg	130	7
4	CVA	74	0	pos	102	6
5	CVA	87	7	pos	58	11
6	Familial APS	0	0	neg	46	2
7	CVA	86	4	pos	24	3
8	Upper limb dystonia, CVA	97	10	pos	83	2
9	CVA	60	0	pos	53	7
10	SLE, VTE	85	0	pos	59	2

Table 17 Summary of the patients providing samples for purification of IgG fractions.
Mean levels for aCL and anti-β2GPI titres are shown

Chapter 4

An investigation of the interaction of β2-glycoprotein I with plasmin and other proteolytic enzymes

4.1 Introduction

β2GPI undergoes a specific proteolytic cleavage in domain V by plasmin. The effect of plasmin on domain V significantly reduces the ability of the protein to interact with negatively charged phospholipid surfaces, by cleaving the hydrophobic loop crucial for this interaction. It is also likely that the positively charged residues flanking the loop are disrupted by this process. Other enzymes have been reported to cleave β2GPI in the same manner including Xa (Ohkura *et al*, 1998), trypsin, elastase (Horbach *et al*, 1999) and recently XIa (Shi *et al*, 2005).

Within this section of work, I undertook to examine whether the processing of β2GPI by plasmin could be modulated by the presence of antiphospholipid antibodies from patients with APS. This involved setting up a system to both detect and quantify the amount of cleavage of β2GPI by plasmin. To do this I employed the reduction of binding of β2GPI to cardiolipin, which takes place on cleavage of domain V by plasmin. This involved setting up an assay for β2GPI binding to cardiolipin; this assay was also used to look at the binding of different polymorphic variants of β2GPI to phospholipid surfaces. The effect of these polymorphisms on proteolytic processing of the protein was also examined. Because some of these polymorphisms affect binding of β2GPI to phospholipid themselves, SDS PAGE was used to follow the cleavage of β2GPI over time in these cases.

The work in this section also examined the effect of two other protease enzymes on β2GPI: plasma kallikrein and factor Xa. There are many reports of interactions between the contact system and the antiphospholipid syndrome and it was therefore interesting to see whether kallikrein could proteolytically interact with β2GPI. Moreover, kallikrein has pro-fibrinolytic effects, and recent work showing that clipped β2GPI can impede the activation of plasminogen would allow the possibility of a negative feedback loop if kallikrein were able to process β2GPI in a similar way to plasmin. The literature is at variance as to whether β2GPI is clipped by Xa (Horbach *et al*, 1999) (Ohkura *et al*, 1998), I therefore decided to investigate the effect of this protein on β2GPI for myself.

4.1.1 Background

As discussed in the introductory sections, the structural motifs of domain V of β2GPI are crucial to the interaction of the protein with negatively charged surfaces including phospholipid. In the late 1990's, evidence began to emerge that serine protease activity, particularly that of plasmin, can specifically cleave domain V of β2GPI and thereby reduce the binding of the protein to negatively charged phospholipids. Prior to this, descriptions had appeared in the literature describing the detection of "nicked" β2GPI in samples of commercially prepared protein (Hunt *et al*, 1993). This study described two cleavage sites in domain V, a major site between Lys317 and Thr318 and a minor site between Ala 314 and Phe 315.

Further work (Ohkura *et al*, 1998) looking at the effect of various proteases on this region of β2GPI has been carried out. The results of this study make it likely that plasmin is responsible for most of the nicked β2GPI seen *in*

vivo and in the commercial preparation of the protein. Moreover, this effect was observed for β2GPI bound to phospholipid. A very slow cleavage of domain V by factor Xa (also at the Lys317-Thr318 site) was observed in this study, while no effect of thrombin, urokinase, and tPA or tissue factor/VIIa was seen. Interestingly, the workers in this study could not detect the presence of Ala 314-Phe 315 nicked β2GPI only that with cleavage at the Lys317-Thr318 site.

Analysis of the product of heparin-sepharose affinity chromatography for purification of β2GPI reveals the presence of nicked β2GPI (Horbach *et al*, 1999). Analysis of the purification elution peaks in this study revealed three peaks, two of which represented nicked β2GPI (designated β2GPI*1 and β2GPI*2) and a non-cleaved form (designated $\beta2GPI^{intact}$). Although no obvious biochemical differences could be discerned between β2GPI*1 and β2GPI*2, a murine monoclonal antibody raised against nicked β2GPI (13A10) was found to recognise only β2GPI*1. Interestingly, 13A10 was found not to bind β2GPI nicked by plasmin when bound to a high binding elisa plate, the implication being that the form produced here is β2GPI*2. The workers in this paper suggest that in plasma, β2GPI*2 may be immediately converted to β2GPI*1. Perhaps a change in tertiary structure differentiates β2GPI*1 from β2GPI*2. Horbach *et al* also found that levels of nicked β2GPI were elevated *in vivo* in patients with sepsis and DIC and in patients treated with streptokinase compared to healthy controls.

Heparin is often employed in the therapeutics of APS particularly in relation to the obstetric complications of the condition. β2GPI is known to bind to heparin and site directed mutagenesis studies have shown that the Lys residues

in domain V are important to this interaction (Guerin *et al*, 2002). Guerin *et al* demonstrated that heparin is able to increase the cleavage of β2GPI by plasmin. This has led to speculation that heparin may exert some of its effect at the level of β2GPI, firstly by reducing the binding of the protein to phospholipid surfaces and also by increasing the generation of a non-phospholipid binding form of the protein.

4.1.2 Structural and functional changes in nicked β2-Glycoprotein I

Cleavage of the peptide bond between Lys317 and Thr318 would be predicted to cause disruption of the hydrophobic loop motif of domain V of β2GPI (see figure 9). Such a change in structure would explain the decrease in phospholipid binding observed on cleavage of β2GPI. That the phospholipid binding of the protein is exquisitely sensitive to alterations around this motif is highlighted when one observes the reduced phospholipid binding of the protein in the presence of the Thr316Ser and Cys306Gly polymorphisms (discussed in more detail in chapter 6). Moreover, cleavage at the Lys317-Thr318 site will disrupt the conformation of positively charged residues, which flank the hydrophobic loop. A detailed analysis and elucidation of the tertiary structure of clipped β2GPI based on X-ray crystallographic studies is available in the literature (Matsuura *et al*, 2000).

To date there is limited data available as to whether clipped β2GPI has a physiological role. At present, interactions have been demonstrated for clipped β2GPI at the level of factor XI activation by factor XIIa (Shi *et al*, 2004) and in extrinsic fibrinolysis (Yasuda *et al*, 2004). Shi *et al* demonstrated that the ability to inhibit XIIa and thrombin activation of XI possessed by intact β2GPI was lost

upon the protein being nicked by plasmin. The authors speculate that APL may bind to β2GPI preventing this inhibition and thereby increasing clotting factor activation. Yasuda *et al* have demonstrated that nicked β2GPI can bind glu-plasminogen, inhibit binding of glu-plasminogen to fibrin monomer and reduce the activity of newly generated plasmin in a tPA-stimulated system. Thus nicked β2GPI may take part in negative feedback control of local fibrinolytic potential.

4.1.3 Nicked β2GPI in pathology

At the time of writing, there have been only limited studies into whether the presence of aPL can influence the action of plasmin or other proteases on β2GPI. From a pragmatic point of view one can see a conundrum with regard to the role of nicked β2GPI and the prothrombotic state. On the one hand nicked β2GPI appears to inhibit fibrinolysis and increase XI activation (indirectly via loss of inhibitory activity). On the other hand one would imagine, that nicked β2GPI will be less able to interact with aPL at cell surfaces and therefore (by whatever mechanism this occurs by) transduce their pro-inflammatory and prothrombotic effects.

Matsuura *et al* demonstrated that an anti-β2GPI monoclonal antibody with specificity for domain V of β2GPI (Cof-18) was able to inhibit cleavage of this region (Matsuura *et al*, 2000). On the other hand, Itoh *et al* (Itoh *et al*, 2000) have demonstrated increased levels of nicked β2GPI in patients with leukaemia and with lupus anticoagulant activity. Whether this increase in patients with LA activity represents the result on ongoing fibrinolytic activation in subjects with a chronic prothrombotic state remains unanswered. Yasuda *et al* have shown an

increase in nicked β2GPI in patients with ischaemic stroke and lacunar infarcts compared to healthy controls subjects (Yasuda *et al*, 2004).

4.1.4 Aims of this section

1) To examine the effect of aPL on the process of β2GPI cleavage by plasmin.
2) To investigate whether plasma kallikrein and factor Xa can cleave β2GPI in domain V.
3) To examine the effect of genetic polymorphisms in domain V of β2GPI on the cleavage of domain V by plasmin.

4.2 An investigation into the effect of aPL on plasmin mediated cleavage of β2GPI

4.2.1 Preliminary experiments on the cleavage of β2GPI by plasmin

In order to explore the process of cleavage of β2GPI by plasmin (and other enzymes), I needed to set up an experimental system, which could cause cleavage of β2GPI and then detect the process. Initially, I set up a simple test system with human plasmin (Enzyme Research) final concentration 50μg/ml in PBS added to platelet poor plasma from a volunteer and a control tube with no plasmin added (with buffer to the same volume) incubated overnight at 37°C. Plasma and plasmin in PBS at 100μg/ml were mixed in equal volumes. In this untreated system only a very slight reduction in β2GPI binding to cardiolipin could be observed when this was assayed for test and control samples using the β2GPI cardiolipin binding assay the method for which is described in the general methods section (figure 16).

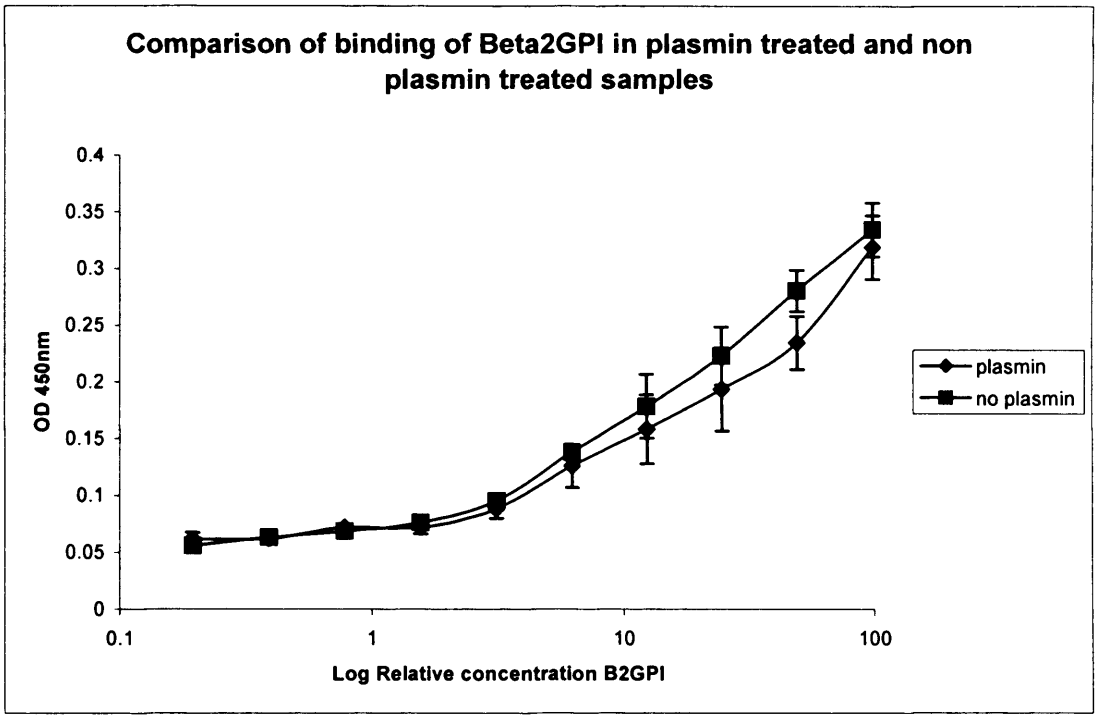


Figure 16 Comparison of binding of β2GPI from plasmin treated and non treated plasma samples.

For each sample, the starting dilution was 1 in 100 in PBS followed by 9 doubling dilutions. Results of the mean and standard deviations are shown for quadruplicate wells. No acetone pre-treatment of plasma samples was undertaken for this experiment. The above result is representative of duplicate experiments.

In this system, it was apparent that the action of the plasmin was impeded – a likely explanation for this was the presence of Serpin activity (including alpha2-plasmin inhibitor) in the plasma. In an attempt to reduce this effect, I pre-treated the plasma samples with acetone.

Acetone treatment of plasma has been used in a variety of amidolytic substrate assays of haemostatic components, in particular assays of the contact system (Gallimore *et al*, 1987;Hoem *et al*, 1989). Under suitable conditions, acetone removes the known inhibitors of factor XIIa and kallikrein (Briseid & Johansen, 1983;Nakahara, 1980) and considerably enhances factor XIIa activity, which can be completely inhibited by corn trypsin inhibitor (Hojima *et al*, 1980;Gallimore *et al*, 1987;Hoem *et al*, 1989). Since the major inhibitors of

factor XIIa and kallikrein are the serpins: C1-esterase inhibitor, antithrombin, alpha-1-antitrypsin and heparin dependant protein C inhibitor, it is likely that their function is being compromised. The most likely mechanism is the irreversible oxidation of amino acid residues in the reactive centre of the serpins, which has been demonstrated for alpha-2-plasmin inhibitor, antithrombin, alpha-1-antitrypsin and C1-esterase inhibitor, with oxidants such as chloramine T, N-chlorosuccinimide, ammoniumperoxodisulphate and hydrogen peroxide (Stief *et al*, 1988).

The acetone treatment procedure was as follows: plasma samples were mixed with acetone in a ratio of one part acetone to three parts plasma and vigorously mixed; samples were cooled at 4°C for 15 minutes and centrifuged at 12,000g for three minutes; the supernatant was then collected for use. Results comparing the β2GPI binding to cardiolipin following plasmin treatment (and control with buffer only added) overnight in acetone treated and non-treated samples are shown in figure 17. Assay conditions were otherwise as above.

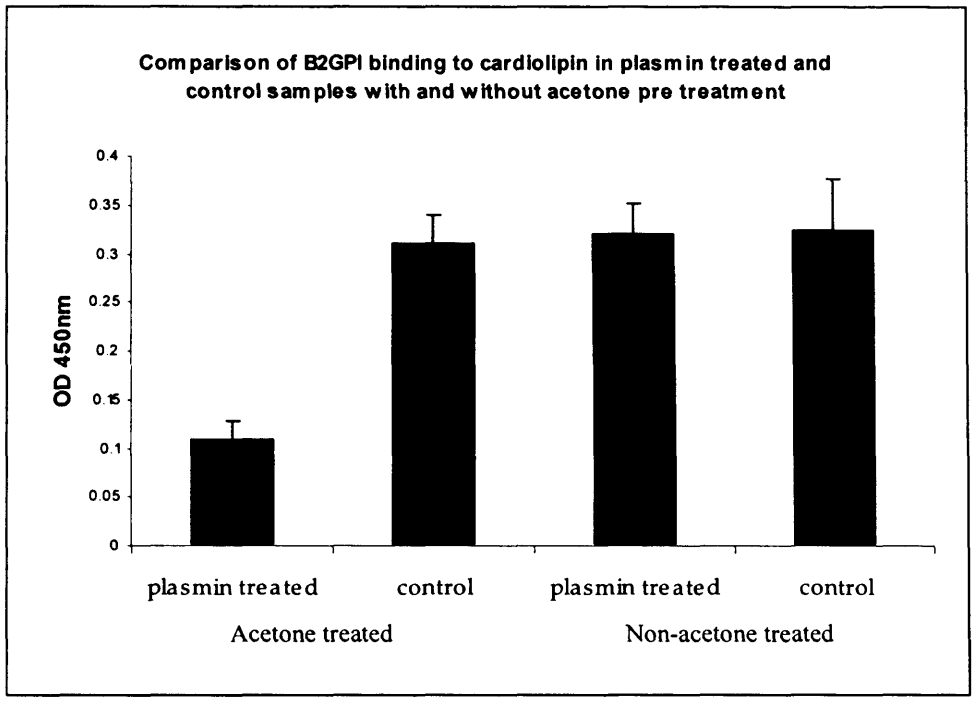


Figure 17 Comparison of β2GPI binding in acetone treated and non treated plasma samples after incubation with plasmin.

Results and standard deviations are shown for quadruplicate wells in the β2GPI cardiolipin binding assay. Samples were diluted 1 in 200 for the binding assay.

4.2.2 Determining the time course of the plasmin cleavage reaction

In order to develop a "working assay" for analysis of the interaction of this process with aPL I wanted to shorten the reaction time used (to try and "catch" the process prior to maximum cleavage where any differences may have been missed) and develop a way of stopping the reaction so samples could be saved after plasmin treatment prior to assay in the cardiolipin binding system. I chose to use a combination of Aprotinin and rapid cooling to stop the reaction. To develop this idea I set up a time course experiment.

For this work lyophilised reference plasma was used (Technoclone® Coagulation Reference) made up as per the manufacturer's instructions and pre-

treated with acetone as described above. The plasma was then mixed with an equal volume of plasmin (in PBS); again the final concentration of plasmin was 50μg/ml. The reactant mix was incubated at 37°C and 50μl aliquots removed at time 0, 30, 60 and 90 minutes. The aliquots were taken into 10μl of Aprotinin (10,000 KIU/ml), mixed and immediately frozen to -70°C for later analysis. The stored samples were then assayed for β2GPI cardiolipin binding at a dilution of 1 in 200. Results are illustrated in figure 18.

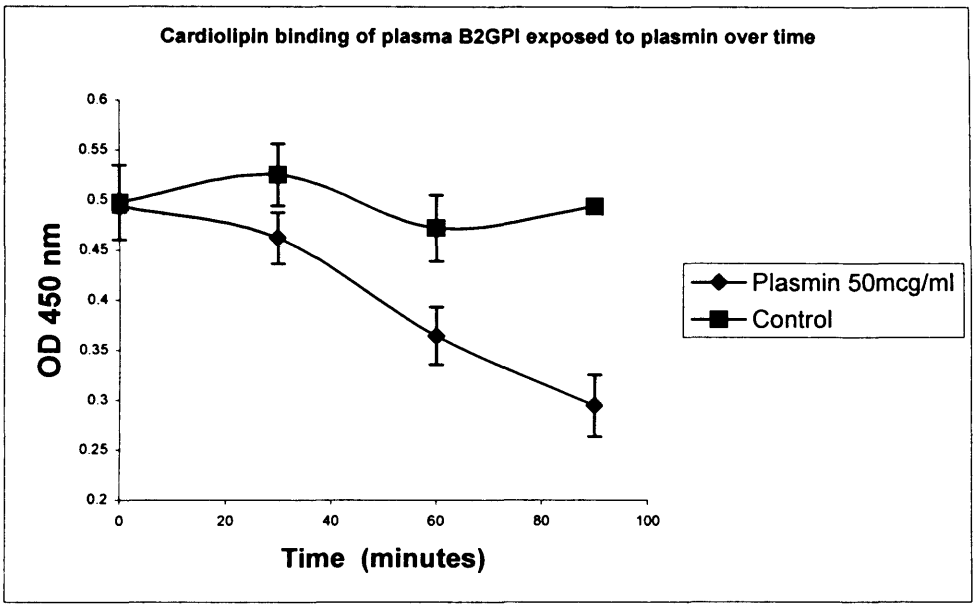


Figure 18 Time course of decrease in cardiolipin binding of β2GPI over time on incubation with plasmin.

Results show the mean and standard deviation of quadruplicate wells in the cardiolipin-binding assay.

I also ran time course experiments for plasmin at lower concentrations (5, 0.5 and 0.05 μg/ml. No significant reduction in β2GPI binding to cardiolipin was observed for these concentrations over the time course studied.

4.2.3 Preliminary experiments on the effect of APL on plasmin mediated cleavage of β2GPI

While the process and molecular details of the proteolysis of domain V of β2GPI have been extensively investigated and discussed in the literature, the effect of the presence of aPL on the process had not. One paper has reported that a monoclonal anti-β2GPI antibody with specific activity against domain V (Cof18) can impede cleavage of β2GPI by plasmin (Matsuura *et al*, 2000). I wanted to see what effect the presence of aPL could have on the process.

For this work, purified IgG fractions from patients with APS and pooled normal plasma were prepared in PBS as described in the materials and methods section. In these experiments, the addition of the IgG fraction to plasma necessitated its dilution however the reactant was constructed such that the plasmin in the assay was diluted to the same extent. Therefore although the plasmin concentrations in the following experiments are lower than that in the preceding ones, the enzyme: substrate ratio (plasmin:β2GPI) remains the same.

Reactions were set up in microcentrifuge tubes with 100μl volumes each of acetone treated pooled normal plasma (Technoclone®); IgG fraction (from an APS patient with high titre aPL (Patient 3) or pooled normal plasma) in Dulbeco's PBS (final concentration 0.5mg/ml); plasmin in PBS (final concentration 33μg/ml). For each arm of the experiment control tubes with no plasmin were set up. Tubes were incubated for one hour at 37°C before aliquots were taken and treated with aprotinin and freezing to -70°C as described above. The samples were then run in the cardiolipin binding assay to quantify the decrease in cardiolipin binding in the aPL exposed sample compared to that in

the non APL treated sample. The results of this preliminary experiment are shown in figure 19.

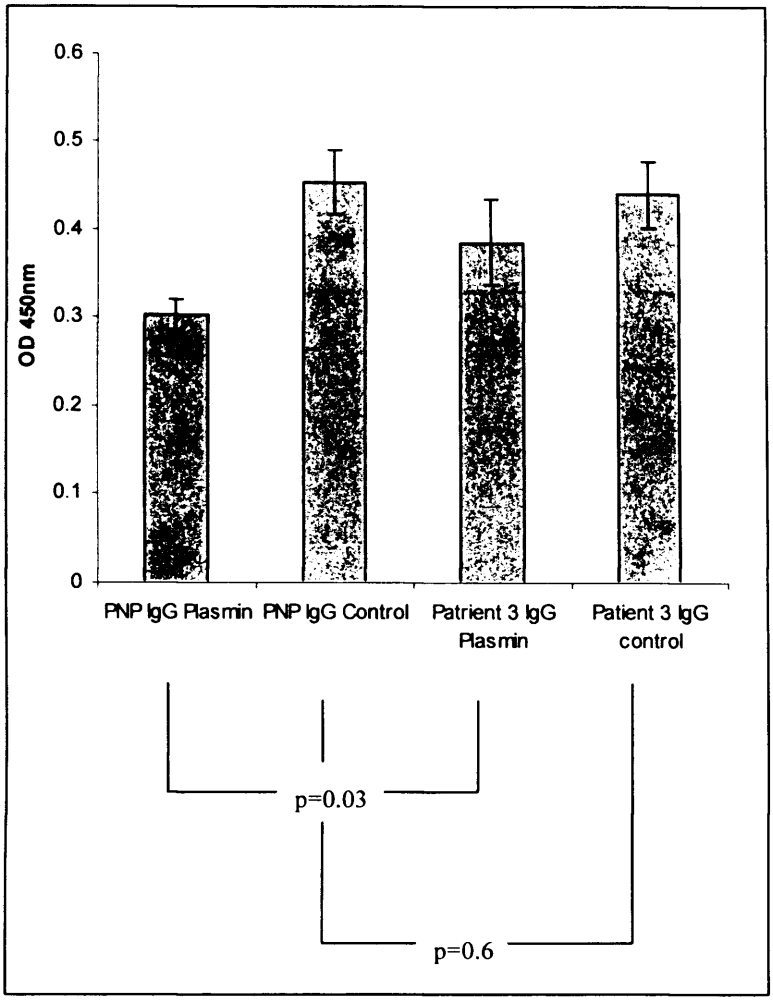


Figure 19 Cardiolipin binding for plasma β2GPI treated with plasmin in the presence of aPL (Patient 3 IgG) and pooled normal IgG.

Samples were diluted 1 in 200 for the cardiolipin binding assay. The mean and standard deviation for quadruplicate wells are shown. 2 tailed independent sample t-test p values are shown comparing plasmin treated and control arms. (No significant difference was observed between plasmin and non-plasmin treated arms in the aPL exposed samples).

The lack of difference in binding between the control arms of the experiment suggested that the presence of APL in the assay was not causing impairment or augmentation of β2GPI binding to phospholipid. There was a small but significant difference in binding between the aPL treated and a non-

aPL treated plasmin exposed arms suggestive of a reduction in plasmin-mediated cleavage in the APL treated sample. To further test this phenomenon prior to a larger scale set of experiments, I tested this high titre APL patient IgG fraction (Patient 3) in a repeat of the above experiment on two separate occasions. A similar reduction in the apparent amount of plasmin mediated cleavage of β2GPI was observed for the aPL exposed sample (figure 20).

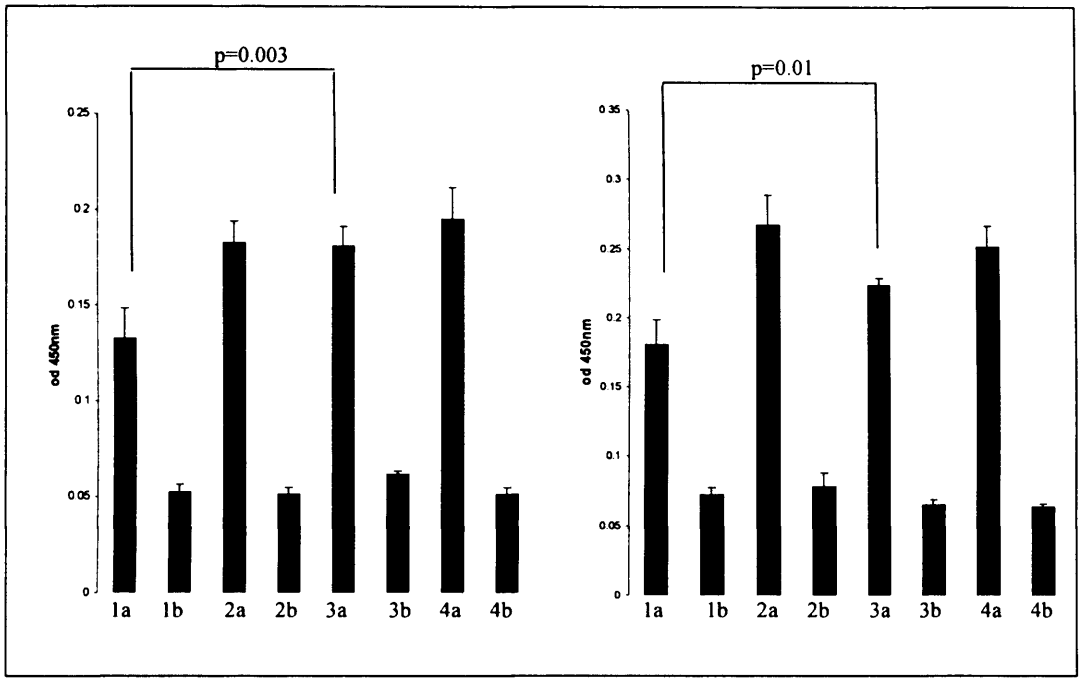


Figure 20 Cardiolipin binding for plasmin treated and control plasma β2GPI in the presence of APL (Patient 3) and normal IgG fractions.

A significant difference in binding was observed in the binding between plasmin treated arms in the presence and absence of aPL. The mean and standard deviations of quadruplicate wells in the cardiolipin binding assay is shown (samples diluted 1 in 400). The results of two separate experiments are illustrated. For comparison the binding of β2GPI to non-cardiolipin coated (blank) wells is shown. Two tailed t-tests revealed a significant difference in binding between aPL and non aPL treated samples. There was no difference in binding between the aPL and non-aPL control arms (p value not shown). Key: 1a PNP IgG + plasmin; 2a PNP IgG control; 3a Patient 3 IgG + plasmin; 4a Patient 3 IgG control. 1b-4b = non-cardiolipin well coated results corresponding to 1a-4a.

4.2.4 An examination of the effects of six aPL positive IgG fractions on the proteolysis of β2GPI by plasmin

To further investigate the effect of aPL on the plasmin mediated cleavage of β2GPI, I set up a series of experiments looking at this effect with six different aPL containing IgG fractions. IgG fractions on these patients were isolated as discussed in the materials and methods section. The clinical presentations of the patients examined and their pattern of aPL positivity is shown in table 18.

Patient	Clinical Presentation	aCL IgG	IgM aCL	LA	anti B2GPI IgG %	Anti B2GPI IgM %
1	Multiple VTE	84	42	+	191	52
2	VTE, Lower limb ischaemia	100	7	+	115	15
3	Familial APS, cognitive problems	53	17	-	130	7
4	CVA	74	0	+	102	6
5	CVA	87	7	+	58	11
6	Familial APS	0	0	-	46	2

Table 18 Clinical presentations and aPL positivity patterns of the patients investigated in this section.

aCL IgG and IgM measured in GPLU and MPLU respectively. Anti β2GPI results expressed as percentage of index serum. (Cut offs for aCL and anti-β2GPI levels are given in the general methods section). Mean aPL levels for each patient are shown.

The anti-β2GPI activity of each of the patient and pooled normal plasma IgG fractions were determined using a modification of the anti-β2GPI assay described in the methods section, where a 1 in 10 dilution of IgG fraction at a starting concentration of 1mg/ml was assayed (in contrast to the usual 1 in 100 dilution of patient serum). I chose this concentration change based in the observation that the IgG fractions IgG concentration was approximately 1/10 of that in normal human serum. The results of this assay are shown in figure 21.

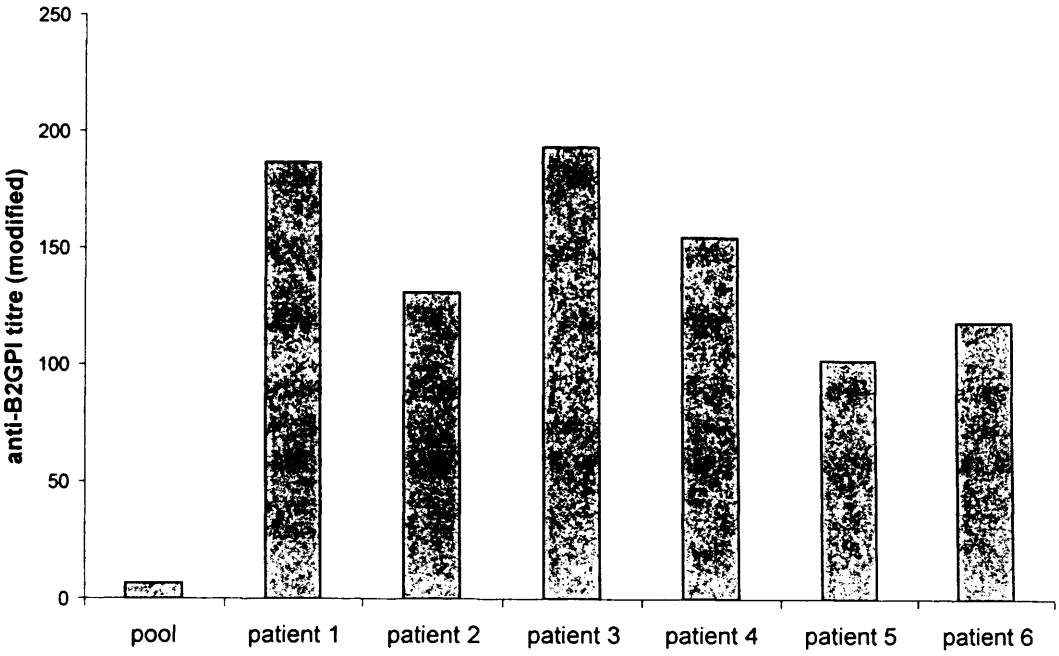


Figure 21 Anti-β2GPI titres for IgG fractions used in this section

The modifications made to the assay are discussed in the text. (Pool = pooled normal plasma IgG fraction).

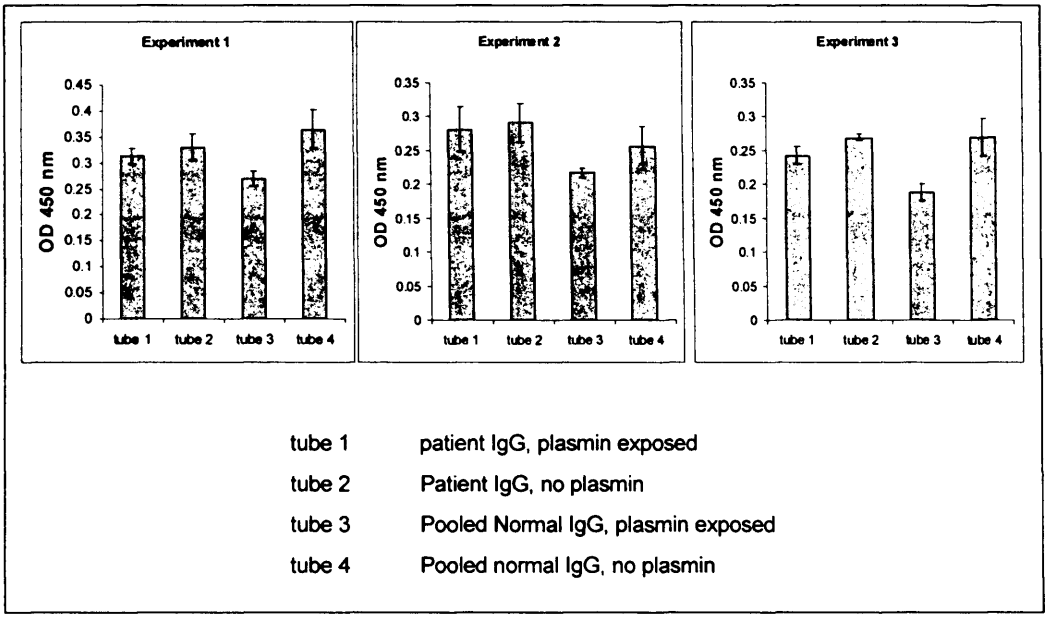
For these experiments, I focused on patients with IgG fractions manifesting a high titre (>100) anti-β2GPI activity in the modified anti-β2GPI assay. The samples of patient IgG used in this set of experiments were therefore a selected group with high levels of anti β2GPI activity.

For each patient, triplicate experiments were set up as per the preliminary experiments described above to compare the effect of the aPL positive IgG fractions compared to IgG from pooled normal plasma. Triplicate test and control tubes were set up containing 100μl volumes of: Acetone treated pooled normal plasma (Technoclone®); IgG fractions from APS patients or pooled normal plasma in Dulbeco's PBS (final concentration 0.5mg/ml); Plasmin in PBS or buffer alone (for control tubes) (final concentration plasmin 33μg/ml). Reagents

were kept on ice prior to incubation at 37°C for one hour. As previously at the end of one hour, an 100μl aliquot was taken into 20μl of aprotinin (10,000 KIU/ml) and frozen to -70°C prior to assay for β2GPI binding to cardiolipin.

The triplicate samples from each experimental run were then assayed for β2GPI binding to cardiolipin as before. In each case test and control samples were diluted 1 in 400 in PBS for analysis of cardiolipin binding; each sample was assayed in quadruplicate wells on the ELISA plate. If the % coefficient of variation for quadruplicate wells exceed 10%, I allowed removal of one point from a quadruplicate data set. If in spite of this, the CV remained higher than 10%, the ELISA was repeated.

To compare results for cardiolipin binding for aPL treated and non-aPL treated samples, I chose to compare the ratio between mean optical densities for quadruplicate wells between the plasmin and non-plasmin treated arms of each experiment. This was done in an attempt to "internally-normalise" the data. Using the raw optical density values for comparison would mean that any change in cardiolipin binding by β2GPI induced by the presence of aPL per se would not be controlled for. (For example, aPL could have increased the affinity for β2GPI to the phospholipid by dimerising the molecule or reduced binding by holding the β2GPI in an immune complex. In reality however, no such effect was observed). Using a ratio also allowed ease of comparison of results from different cardiolipin coated plates. A worked example for one of the patients is shown in figure 22. The results for all six aPL samples are shown in figures 23 and 24.



aPL IgG results	Plasmin	Control	Cardiolipin-Binding ratio
Experiment #1	0.312	0.331	0.945
Experiment #2	0.281	0.291	0.968
Experiment #3	0.243	0.270	0.902
		mean	0.938
		SD	0.034
		%cv	3.586

PNP IgG results	Plasmin	Control	Cardiolipin-Binding ratio
Experiment #1	0.270	0.364	0.742
Experiment #2	0.217	0.257	0.845
Experiment #3	0.188	0.270	0.696
		mean	0.761
		SD	0.076
		%cv	9.983

Figure 22 An example set of triplicate experiments comparing the effect of plasmin on β2GPI binding to cardiolipin in the presence of aPL IgG and IgG from pooled normal plasma.

The graphical data illustrates the results of the β2GPI cardiolipin-binding ELISA assay. The mean and standard deviations for quadruplicate wells are shown. (%CV= % coefficient of variation). (Cardiolipin binding ratio = mean od plasmin treated / mean od control).

Cardiolipin binding ratios	Patient 1 IgG	Pool IgG
Experiment 1	0.945	0.742
Experiment 2	0.968	0.845
Experiment 3	0.902	0.696
mean	0.938	0.761
standard deviation	0.034	0.076
%CV	3.586	9.983

Cardiolipin binding ratios	Patient 4 IgG	Pool IgG
Experiment 1	0.836	0.831
Experiment 2	0.832	0.852
Experiment 3	0.906	0.823
mean	0.858	0.836
standard deviation	0.042	0.015
%CV	4.842	1.802

Cardiolipin binding ratios	Patient 2 IgG	Pool IgG
Experiment 1	0.866	0.780
Experiment 2	0.872	0.868
Experiment 3	0.970	0.841
mean	0.902	0.830
standard deviation	0.059	0.045
%CV	6.511	5.424

Cardiolipin binding ratios	Patient 5 IgG	Pool IgG
Experiment 1	0.865	0.878
Experiment 2	0.927	0.833
Experiment 3	0.911	0.816
mean	0.901	0.843
standard deviation	0.032	0.032
%CV	3.563	3.781

Cardiolipin binding ratios	Patient 3 IgG	Pool IgG
Experiment 1	0.880	0.769
Experiment 2	0.934	0.850
Experiment 3	0.958	0.793
mean	0.924	0.804
standard deviation	0.040	0.042
%CV	4.307	5.186

Cardiolipin binding ratios	Patient 6 IgG	Pool IgG
Experiment 1	0.914	0.791
Experiment 2	0.884	0.794
Experiment 3	0.928	0.810
mean	0.908	0.798
standard deviation	0.023	0.011
%CV	2.483	1.321

Mean Cardiolipin Binding ratios			
aPL Sample	Patient IgG	Pool IgG	p value
Patient 1	0.938	0.761	**0.0208**
Patient 2	0.902	0.830	0.1635
Patient 3	0.924	0.804	**0.0228**
Patient 4	0.858	0.836	0.4353
Patient 5	0.901	0.843	0.0884
Patient 6	0.908	0.798	**0.0016**

Figure 23 Results for cardiolipin-binding ratios for all six aPL IgG fractions and the paired pooled normal plasma IgG fraction.

The cardiolipin binding ratio calculation is illustrated in figure 22. The p-values for a 2 tailed independent samples t test comparing the mean cardiolipin-binding ratios for each aPL IgG and pooled IgG are shown in the lower table. Significant p-values are shown in bold.

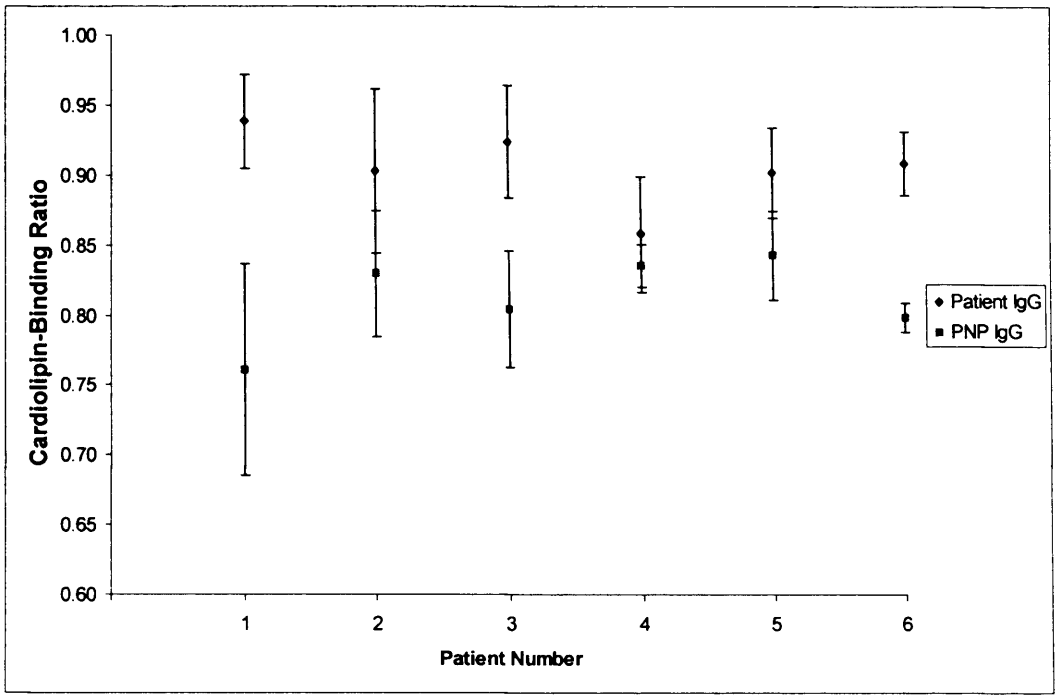


Figure 24 Cardiolipin-binding ratio results for plasmin treated β2GPI in the presence of aPL and pooled normal plasma IgG fractions.

Mean and standard deviations are shown for triplicate experiments. A significant difference was observed for patient samples 1, 3 and 6. The mean, standard deviation and % coefficient of variations for both groups are shown in figure 23.

The results thus indicate a significant (but small) reduction in the plasmin mediated cleavage of β2GPI in the presence of aPL from patients 1, 3 and 6. The experiment described above was also performed on patient IgG fractions with low titre aPL (IgG aCL 6GPLU) and a patient with aPL secondary to leprosy. Both of these patients had modified anti-β2GPI assay titres of less than 100. No significant change in the observed reduction of β2GPI binding to cardiolipin post plasmin treatment was observed for these samples (results not shown).

To examine this apparent reduction in cleavage of β2GPI by plasmin in the presence of aPL further, I repeated the plasmin cleavage experiments on some of the patient samples used above – this time however I used purified

β2GPI rather than pooled plasma. I examined this effect using IgG fractions from two patients who had shown no significant effect in the preceding experiments and one who had. Triplicate test and control reactions were set up as follows: pooled normal plasma IgG or APS patient IgG (final concentration 0.5μg/ml); plasmin in (final concentration 33μg/ml); β2GPI (Scipac®, final concentration 65μg/ml). Reactions were carried out in 10% bovine albumin PBS buffer. Reagents were kept on ice prior to incubation for one hour at 37°C, 50μl aliquots of reactant were taken into 10μl of aprotinin (10,000 KIU/ml) and frozen to -70°C prior to analysis in the cardiolipin-binding assay. Samples were diluted 1 in 400 for the cardiolipin-binding ELISA. This assay was performed on samples from patient 2, 5, and 6. The results are illustrated in figure 25.

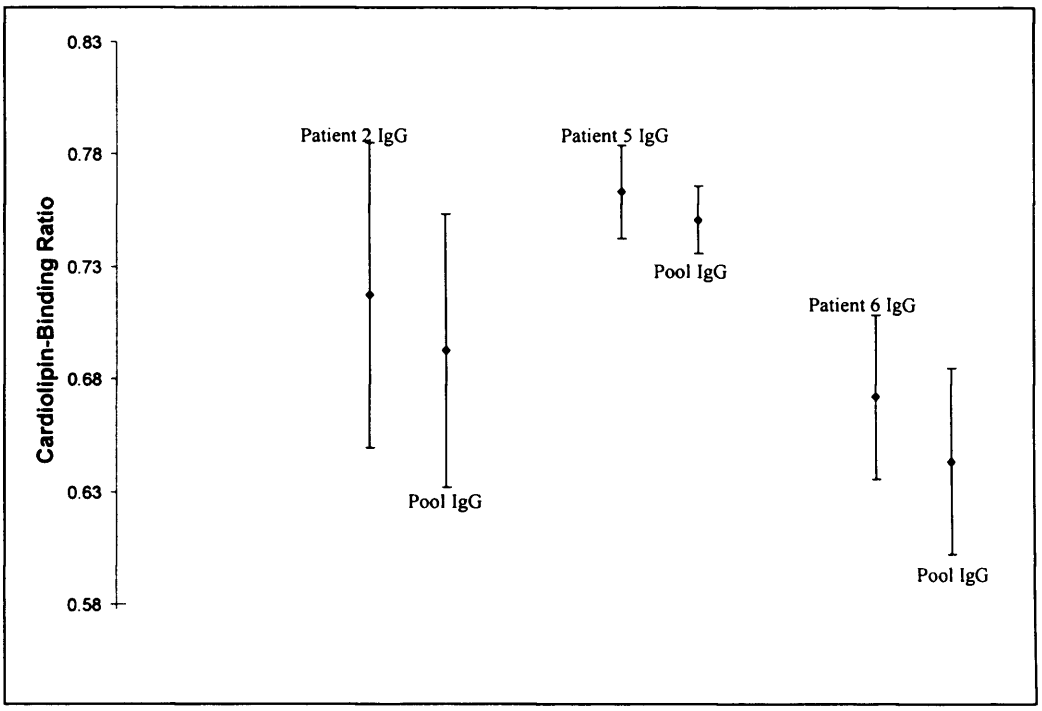


Figure 25 Cardiolipin-binding ratio results for plasmin treated purified β2GPI in the presence of aPL IgG and IgG from pooled normal plasma.

Mean and standard deviations are shown for triplicate experiments. No statically significant difference was found between aPL and pool IgG samples.

In contrast to the reactions carried out with plasma as a source of β2GPI, with purified β2GPI no difference in reduction in cardiolipin-binding was observed between samples exposed to aPL from patient 6 and those not. Again no difference was seen for aPL from patients 2 and 5.

With regard to the experiments performed using plasma as a source of β2GPI, the possibility remained that rather than detecting a change in plasmin activity specific to its action on β2GPI, I was observing a general anti-plasmin activity of aPL. To investigate this I set up an assay using a chromogenic substrate specific for plasmin as follows. Triplicate wells on a non-high binding microtitre plate (96F microplate FX9200 Alpha laboratories) were loaded with reagents on ice as follows: 25μl 2% bovine albumin PBS; 25μl IgG fraction in PBS (final IgG concentration 166μg/ml); 50μl plasmin in PBS (final

concentration 2μg/ml); 50μl plasmin substrate 0.27 mg/ml in water (H-D-Val-Leu-Lys-pNA Channel Diagnostics Ltd). The plate was mixed on a plate mixer and then incubated for 20 minutes. The concentrations of plasmin and substrate had previously shown to be non-rate limiting for substrate concentration over the time period and concentration of plasmin used. The results of this assay are shown in figure 26. With the exception of patient 6, no significant difference in OD was observed here for aPL samples showing an effect of reduced cleavage of β2GPI in the previous experiments.

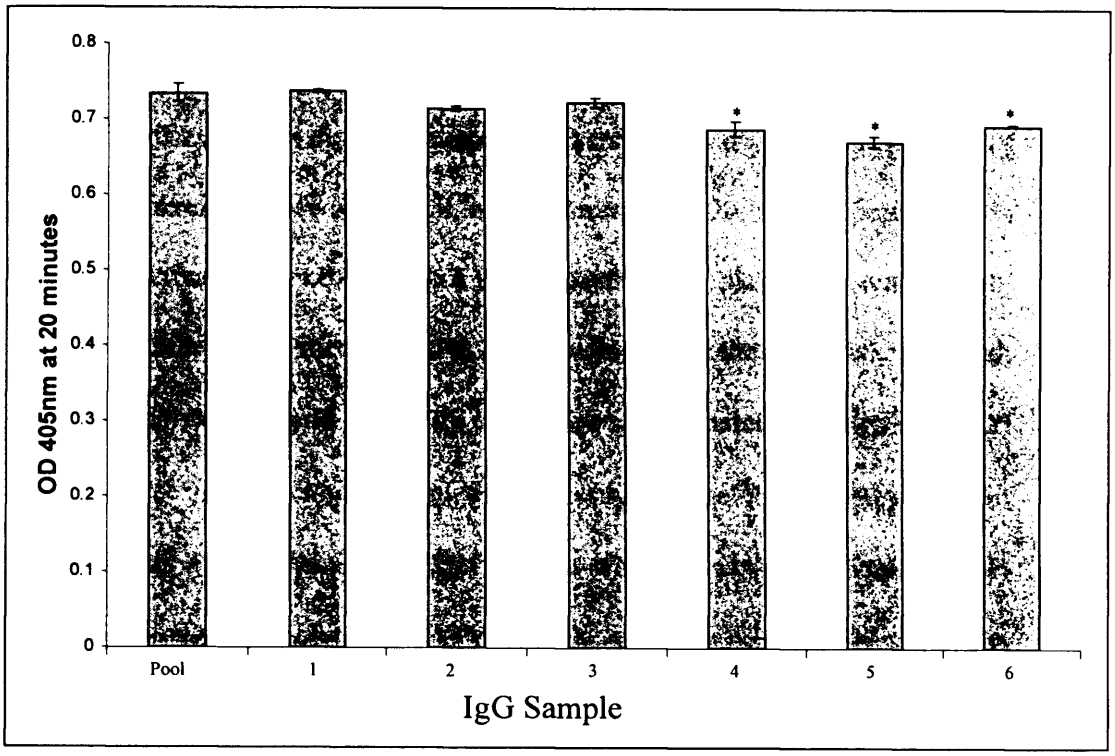


Figure 26 Results of chromogenic substrate assay examining plasmin activity in the presence of pooled normal and aPL positive IgG.

Results show the mean and standard deviations for triplicate wells in the assay. Those marked * showed a $p<0.05$ when compared with the data for the pooled normal IgG using a 2 tailed independent sample t-test.

4.3 An investigation into the potential for kallikrein to cleave domain V of β2GPI

As discussed in the introductory chapters of this thesis, the activation of plasma kallikrein is known to result in a net increase in fibrinolytic potential (via kallikrein activation of uPA and to a lesser extent direct plasminogen activation, as well as bradykinin mediated tPA release from endothelial cells). Although kallikrein activation could indirectly cause plasmin mediated cleavage of β2GPI through these mechanisms, I wanted to investigate whether the enzyme itself could directly affect domain V of β2GPI. The work showing that clipped β2GPI can have an anti-fibrinolytic effect (Yasuda *et al*, 2004) presents the possibility that profibrinolytic enzymes may be involved via clipping of β2GPI in negative feedback on their pro-fibrinolytic activities. At the time of this work no data had been presented as to whether kallikrein could cleave domain V of β2GPI.

4.3.1 Can Kallikrein reduce the binding of β2GPI to cardiolipin?

To answer this question, I set up experiments with purified plasma kallikrein (Enzyme Research) and pure β2GPI (Scipac) or acetone treated pooled normal plasma (Technoclone®) as a source of β2GPI. For the purified protein experiments, reactions were carried out at 37°C for 18 hours in 1% BSA PBS with a β2GPI concentration of 100μg/ml and plasma kallikrein at 50μg/ml. For the reaction with acetone treated plasma, this was added to an equal volume of plasma kallikrein at 100μg/ml in 1% BSA PBS (so final concentration again 50μg/ml) and again incubated at 37°C for 18 hours. For each experiment, control runs with buffer alone, in the absence of kallikrein were included. At the end of the incubation the reactants were taken into aprotinin and frozen to -70°C as

described for the plasmin experiments above. The samples were then assayed for β2GPI binding to cardiolipin at a final reactant dilution of 1 in 400. The results of this initial experiment are shown in figure 27 and illustrate the reduction in binding seen following exposure of β2GPI to kallikrein. With regard to the experiment with plasma the possibility remains that kallikrein could exert its effect through uPA mediated plasminogen activation.

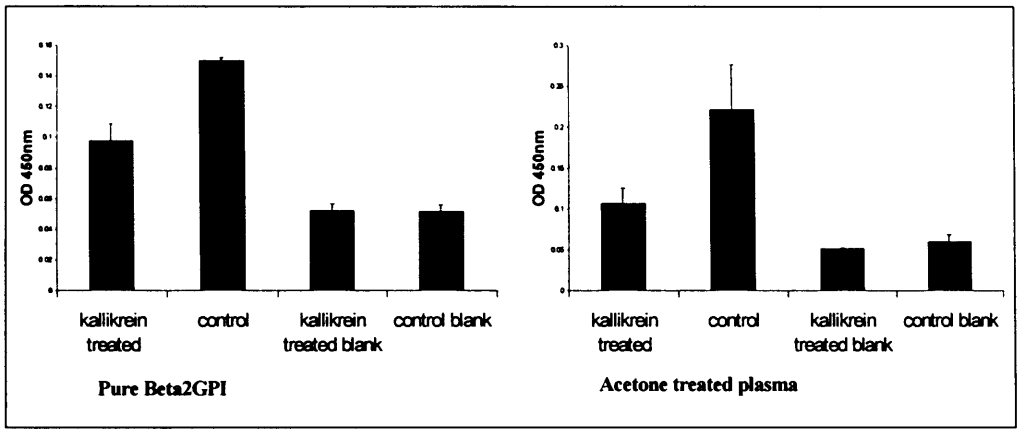


Figure 27 β2GPI binding to cardiolipin for purified β2GPI and plasma β2GPI following treatment with plasma Kallikrein.

Results show the mean and standard deviation for triplicate wells in the cardiolipin-binding assay for test and blank wells (without cardiolipin coating). ($p<0.05$ on 2 tailed t test between kallikrein treated and control arms for both experiments).

The purified β2GPI – kallikrein experiment was repeated in duplicate, in addition to the cardiolipin assay, the reactants from these experiments were also analysed by SDS-PAGE with western blotting for β2GPI as described in the methods section. The results are illustrated in figure 28 and show a second band appearing in the kallikrein exposed β2GPI samples on SDS PAGE.

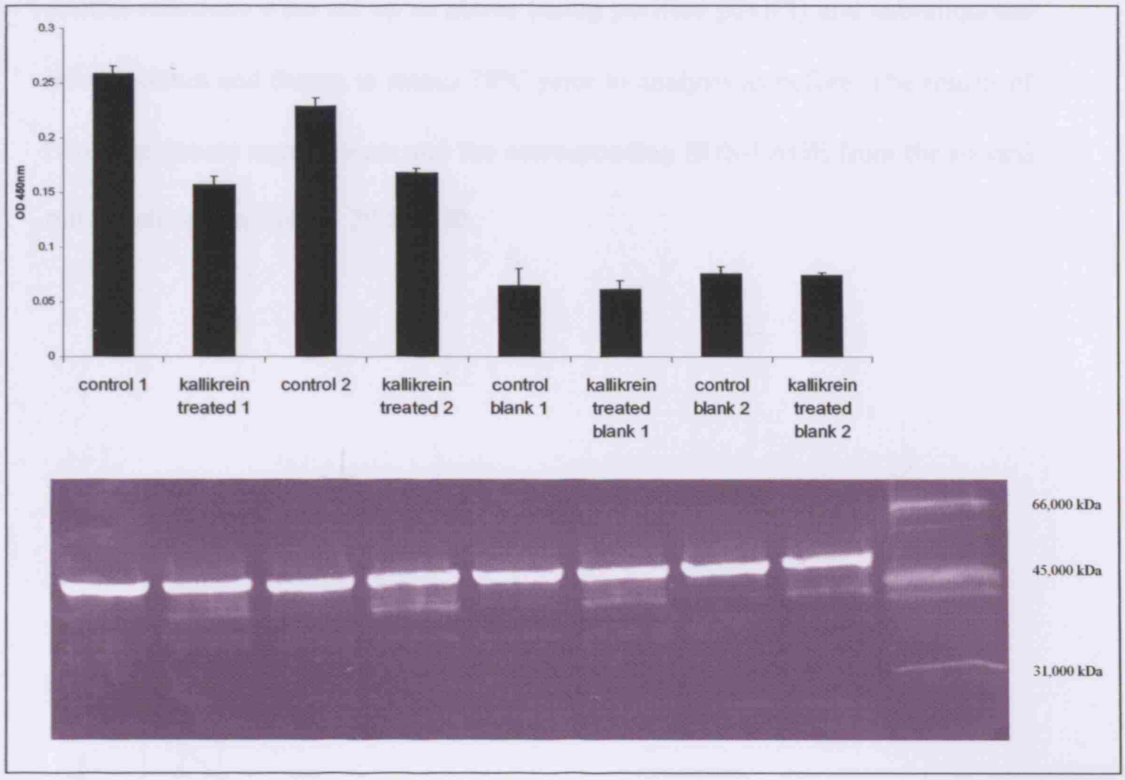


Figure 28 Cardiolipin binding and SDS-PAGE of purified β2GPI treated with plasma kallikrein.

Results for the cardiolipin-binding assay show the mean and standard deviation for triplicate wells (samples at 1 in 400 dilution). A second band corresponding to β2GPI clipped by kallikrein is seen in the kallikrein treated samples on SDS-PAGE (8-16% gradient gel).

Next, I examined the time course of the kallikrein-β2GPI reaction. Test and control reactions were set up as above (using purified β2GPI) and sub-aliquoted into aprotinin and frozen to minus 70°C prior to analysis as before. The results of two time course experiments and the corresponding SDS-PAGE from the second run are shown in figures 29 and 30.

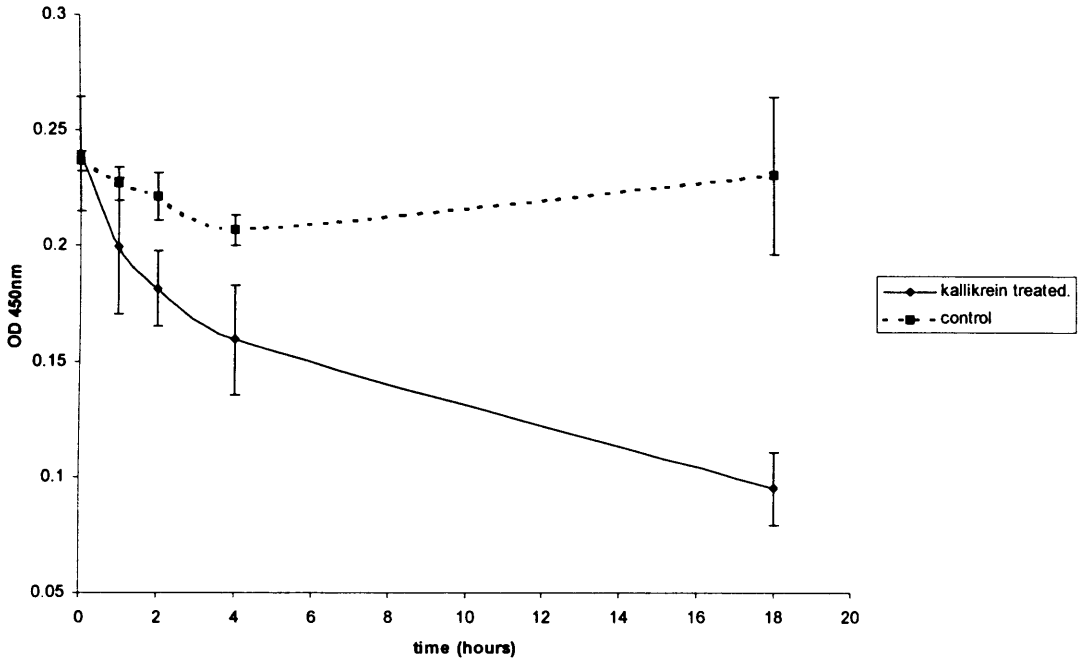


Figure 29 Cardiolipin binding assay results for Kallikrein treated and control β2GPI.

Results illustrate the mean and standard deviations of triplicate wells. Reactions were carried out in 1%BSA PBS; final concentration kallikrein 50μg/ml; final concentration β2GPI (Sci Pac) 100μg/ml.

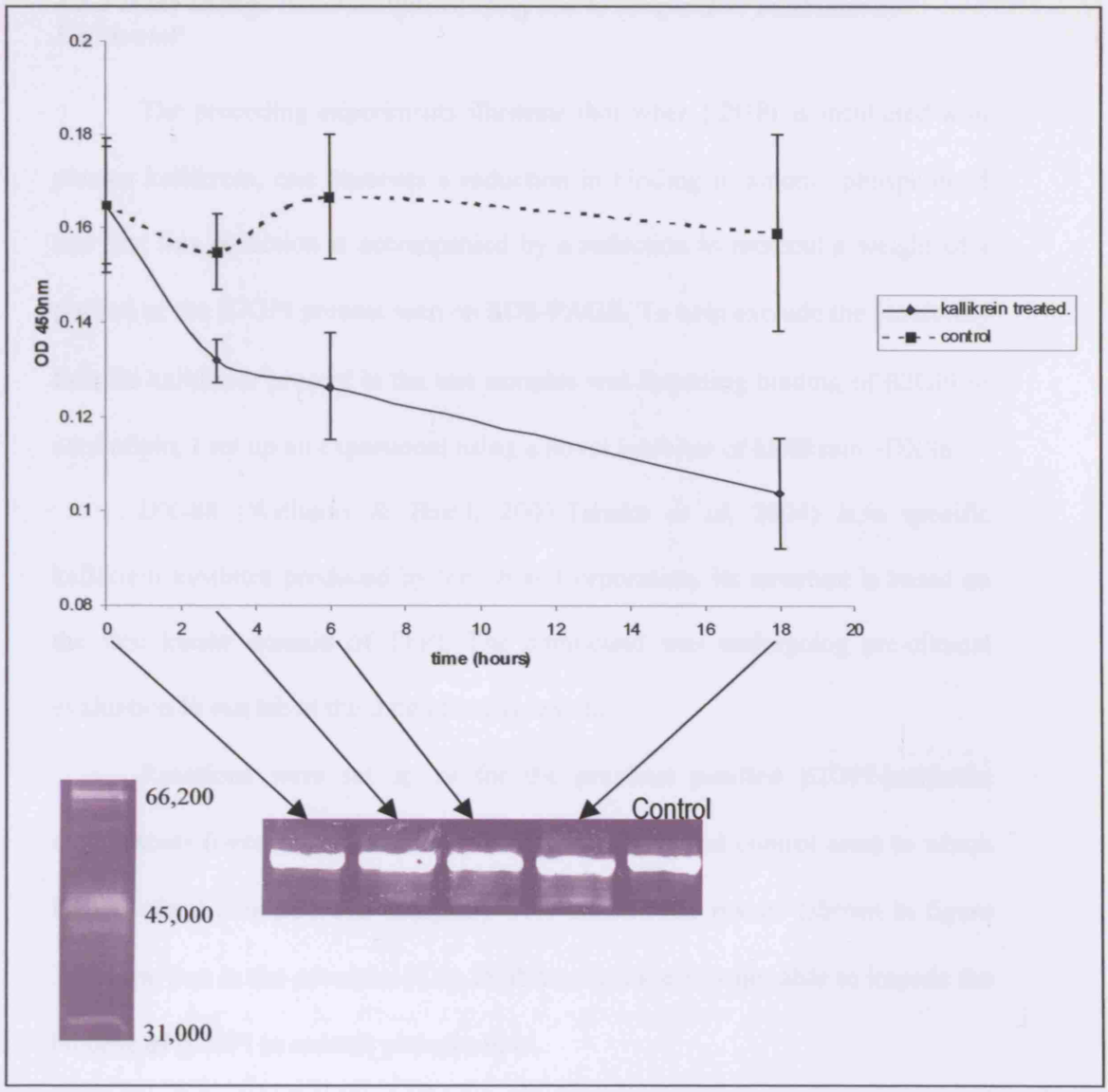


Figure 30 Cardiolipin binding and SDS-PAGE results for kallikrein treated and control β2GPI.

Results illustrate the mean and standard deviations of triplicate wells. Reactions were carried out in 1%BSA PBS; final concentration kallikrein 50μg/ml; final concentration β2GPI (Sci Pac) 100μg/ml. SDS-PAGE on 8-16% gradient gel was performed with western blotting for β2GPI as described in the materials and methods section. Corresponding molecular weight markers for the SDS-PAGE are shown on the left.

4.3.2 Is the change in cardiolipin binding due to competitive inhibition by Kallikrein?

The preceding experiments illustrate that when β2GPI is incubated with plasma kallikrein, one observes a reduction in binding to anionic phospholipid and that this reduction is accompanied by a reduction in molecular weight of a portion of the β2GPI present seen on SDS-PAGE. To help exclude the possibility that the kallikrein present in the test samples was impeding binding of β2GPI to cardiolipin, I set up an experiment using a novel inhibitor of kallikrein –DX88.

DX-88 (Williams & Baird, 2003;Tanaka *et al*, 2004) is a specific kallikrein inhibitor produced by the Dyax Corporation. Its structure is based on the first kunitz domain of TFPI. The compound was undergoing pre-clinical evaluation in our lab at the time of my research.

Reactions were set up as for the previous purified β2GPI-kallikrein experiments (over 18 hours) with two additional test and control arms to which DX-88 (final concentration 10μg/ml) were added. The results (shown in figure 31) show that in the presence of an inhibitor, kallikrein is not able to impede the binding of β2GPI to anionic phospholipid.

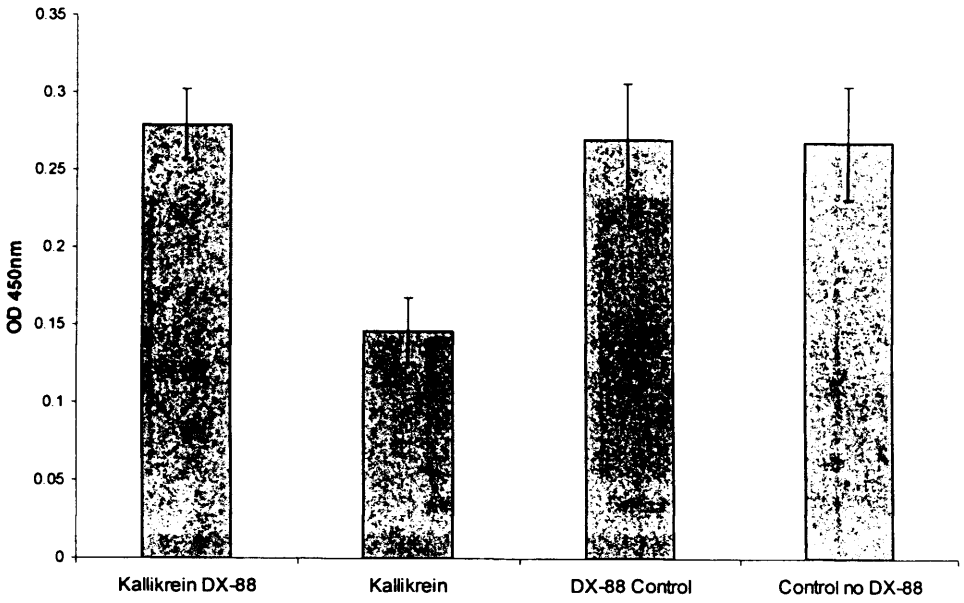


Figure 31 Cardiolipin binding results for purified β2GPI exposed to Kallikrein (18 hours) in the presence and absence of DX-88.

Results show the mean and standard deviation of quadruplicate wells in the cardiolipin binding assay. Samples at 1 in 400 dilution. (Reactions were carried out in 1%BSA PBS; final concentration kallikrein 50μg/ml; final concentration β2GPI (Scipac) 100μg/ml; final concentration DX-88 10μg/ml). Control arms contain no kallikrein.

4.3.3 Is the molecular weight change in β2GPI following Kallikrein exposure the same as that seen following treatment with plasmin and is the product antigenically the same?

To compare the effects of plasmin and kallikrein on β2GPI, I first set up a simple experiment using SDS-PAGE to compare the molecular weight change seen in β2GPI on exposure to each enzyme. The results of this experiment are shown in figure 32. To determine whether the cleaved β2GPI seen following exposure to kallikrein was antigenically the same as that seen following the action of plasmin I used the murine monoclonal antibody 13A10 (Horbach *et al*, 1999). This antibody (the details of which are discussed in the introduction) was kind gift from Prof P. de Groot.

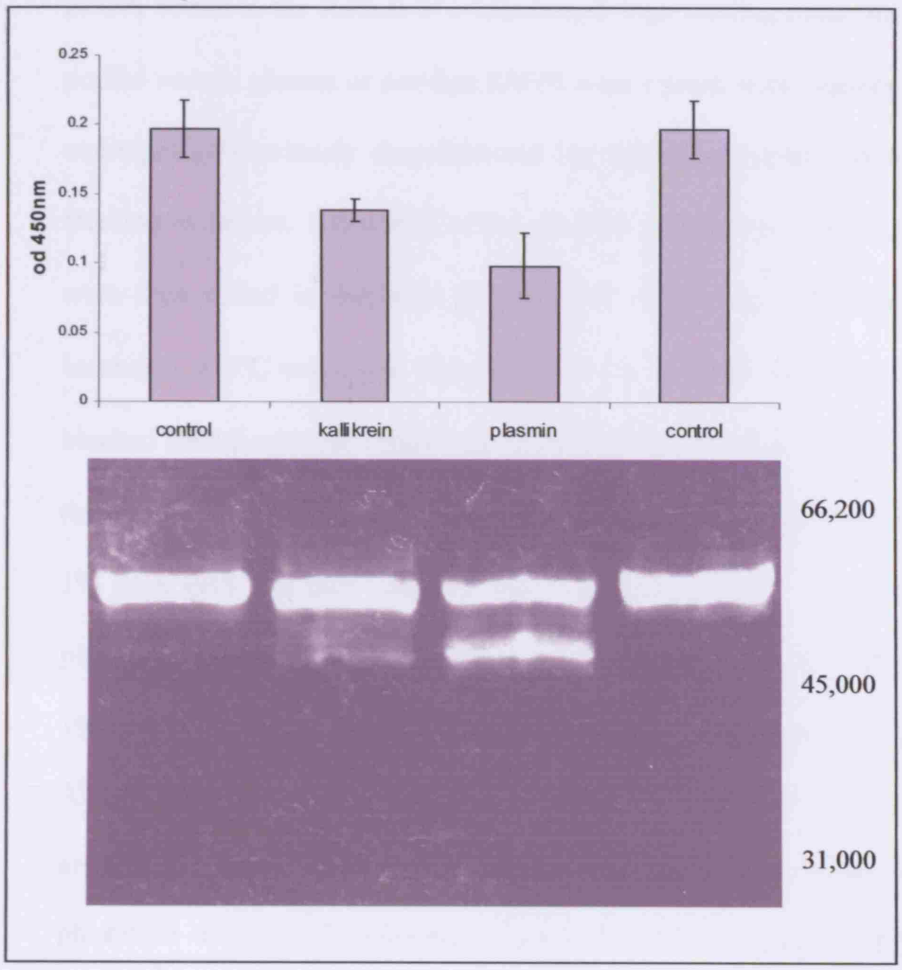


Figure 32 Cardiolipin binding and SDS-PAGE results for purified β2GPI incubated (18 hours) with kallikrein and plasmin

Results for the binding assay show the mean and standard deviation of quadruplicate wells. (Reactions were carried out in 1%BSA PBS; final concentration kallikrein 50μg/ml; final concentration β2GPI (Sci Pac) 100μg/ml; final concentration plasmin 50μg/ml). SDS-PAGE on 8-16% gradient gel. Positions of molecular weight markers (Daltons) are shown to the right.

The results of the SDS-PAGE above show that the molecular weight changes seen when plasmin and kallikrein act on β2GPI is very similar – although the reaction is much less intense with kallikrein. To confirm whether the product was antigenically the same for both reactions, I set up an experiment

looking at binding of the murine monoclonal antibody 13A10 to β2GPI treated with plasmin and kallikrein. Initially, I set up a simple ELISA to detect clipped β2GPI bound to the surface of a Maxisorp® high binding plate. Acetone treated pooled normal plasma or purified β2GPI were treated with plasmin or kallikrein overnight as previously described and the reaction stopped with aprotinin and freezing as before. 100μl/well of the reaction product and control (no protease) were then added in duplicate to the wells of a Nunc Maxisorp® plate and incubated at 4°C overnight. The plate was then washed three times in PBS and blocked for 1 hour with 150μl/well of 1%BSA PBS. The plate was washed again three times with PBS, then 100μl/well of 13A10 (final concentration 10μg/ml) in 1% BSA PBS was then added to each well and incubated for 90 minutes. The plate was washed again with PBS and 100μl/well of a 1 in 1,000 dilution (in 1%BSA PBS) goat anti-murine IgG with alkaline phosphatase conjugate (Sigma A2429) was added to each well and incubated for 90 minutes. After washing, the amount of bound secondary antibody was determined using p-nitrophenyl phosphate disodium hexahydrate (Sigma 104-105) 1mg/ml in diethanolamine buffer as per the anticardiolipin assay described in the general methods chapter. The optical density was read at 405nm.

Results of preliminary experiments using plasmin and acetone treated pooled normal plasma are shown in figure 33.

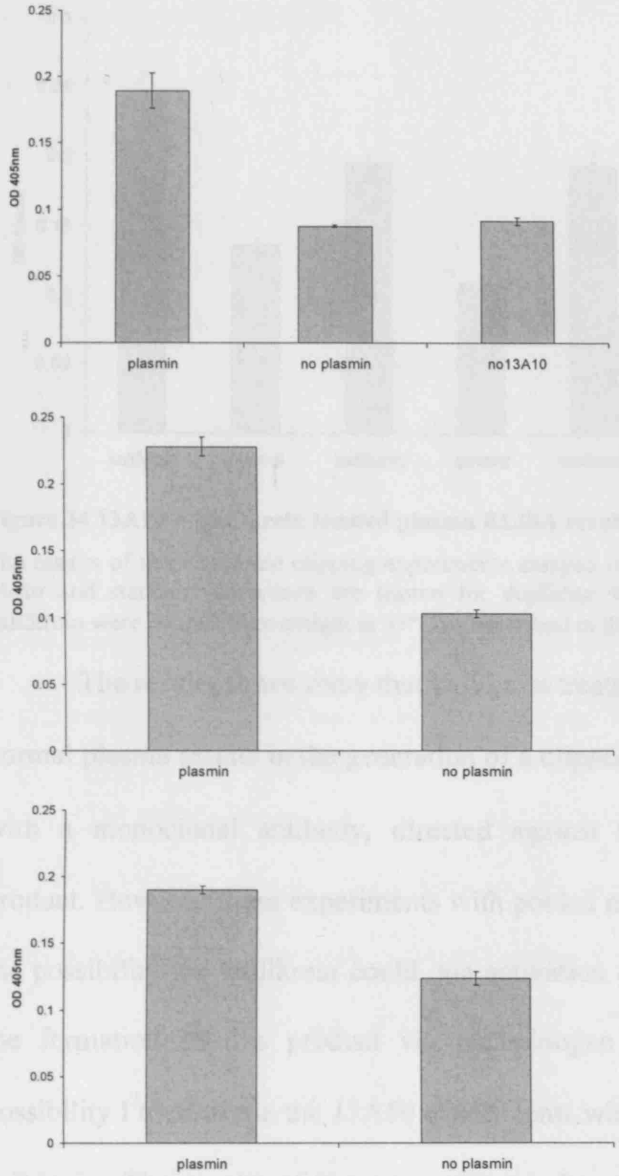


Figure 33 Antibody 13A10 – plasmin clipped β2GPI ELISA results

Figure illustrates the results of 3 separate experiments for plasmin treated pooled normal plasma. Mean and standard deviations are shown for duplicate wells. In the first experiment duplicate wells with plasmin treated sample had buffer added instead of 13A10 (labelled no 13A10). OD 405nm is proportional to binding of antibody 13A10.

Having established that this assay gave an increased signal with clipped β2GPI compared to unclipped β2GPI, I re-ran the assay this time using kallikrein and acetone treated pooled normal plasma. The results are shown in figure 34.

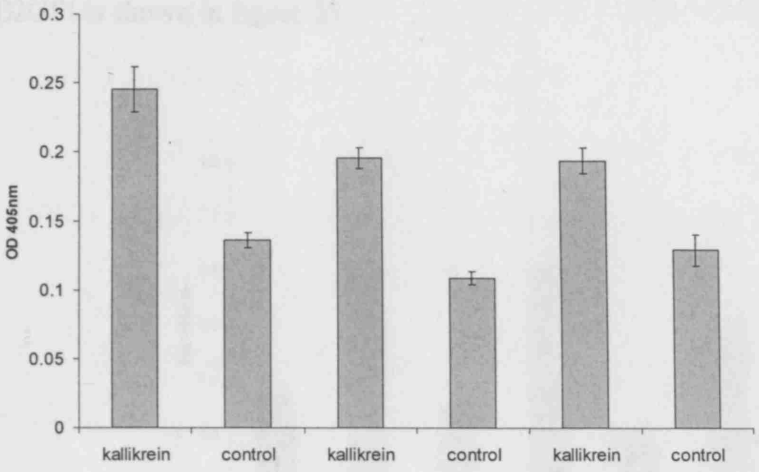


Figure 34 13A10 – Kallikrein treated plasma ELISA results

The results of three separate clipping experiments assayed on the same ELISA plate are shown. Mean and standard deviations are shown for duplicate wells. Acetone treated plasma and kallikrein were incubated overnight at 37°C as described in the text.

The results above show that kallikrein treatment of acetone treated pooled normal plasma results in the generation of a clipped β2GPI species, which reacts with a monoclonal antibody, directed against the plasmin β2GPI reaction product. However these experiments with pooled normal plasma did not exclude the possibility the kallikrein could, via activation of uPA, be indirectly causing the formation of this product via plasminogen activation. To exclude this possibility I tried to run the 13A10 experiments with purified β2GPI and purified kallikrein. The results of these experiments however were not as expected in light of the other data I had already generated and led me to re-design my 13A10 antigen detection method when used with plasma free samples. When the 13A10 ELISA described above was performed with purified proteins, I found that the binding of 13A10 was the reverse of what I would expect, i.e. the binding appeared to be stronger in the control (non-protease) arms. This finding was seen

for both plasmin and kallikrein treated β2GPI. An example for kallikrein treated β2GPI is shown in figure 35.

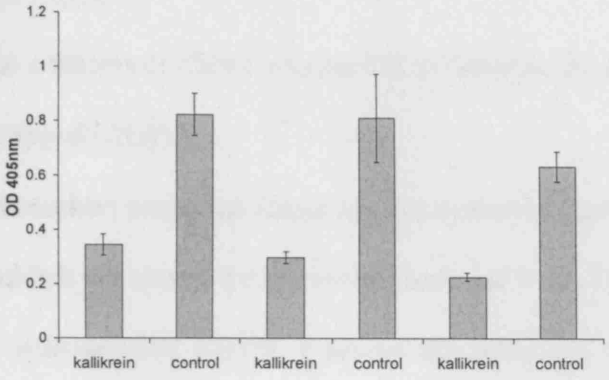


Figure 35 13A10 ELISA results using purified β2GPI and kallikrein

β2GPI-kallikrein incubated overnight in 1% BSA PBS at 37°C as described previously in the text. The mean and standard deviations for duplicate wells are shown.

The results above clearly show that in the non-plasma based system, that the "signal" from 13A10 was greater in the arms with no protease exposure. Possibilities for this somewhat surprising result included:

1) That the purified β2GPI from Scipac contained a species of β2GPI, which reacted with 13A10 but was somehow removed by plasmin/kallikrein treatment. This possibility seemed unlikely in light of the fact that the SDS PAGE of the pure β2GPI showed a clean single band, had normal cardiolipin binding which was in fact lowered on plasmin treatment and gave the same change in band pattern on SDS PAGE as β2GPI from normal plasma when treated with protease.

2) That on binding to the Maxisorp® high binding plate, the pure β2GPI was taking on a conformation (possibly also related to the absence of a plasma cofactor – see 3) also) which had an increased capacity to bind 13A10.

3) That a plasma co-factor was needed to facilitate the binding of 13A10 to clipped β2GPI.

The alternatives presented above are not necessarily mutually exclusive. To try and establish the reason for the results seen and to find a working 13A10 assay for use with purified β2GPI, I set up the following variations on the original assay:

1) As per the original assay, but with the test samples mixed (50:50) after stopping the reaction with aprotinin, with either pooled normal plasma or 5% BSA PBS, prior to coating on a Maxisorp® plate overnight. This was to see whether the presence of a plasma co-factor could influence the binding of 13A10 to the clipped β2GPI product.

2) To see whether the presence of the high binding ELISA plate was in influencing the binding of 13A10, I set up an immune complex capture assay. In this assay (described below in detail), the binding of 13A10 to the protease treated β2GPI was carried out in "liquid phase" rather than at the surface of an ELISA plate.

3) I also tested a sandwich ELISA with 13A10 coated onto a high binding ELISA plate overnight as the first layer.

For all of these experiments, control, plasmin treated and kallikrein treated β2GPI was generated as follows: β2GPI (Scipac) final concentration 50μg/ml; Kallikrein (Enzyme Research) final concentration 50μg/ml / Plasmin

(Enzyme Research) final concentration 50μg/ml / Control with PBS only; reactions carried out in 1%BSA PBS at 37°C for 18 hours. For experiments 1) and 3) reactions were stopped by taking aliquots into aprotinin, for the immune complex capture assay, aprotinin was included in the antibody binding step (see below).

The results of experiment 1) are illustrated in figure 36. The results show that when the reactants are mixed with pooled plasma prior to binding that a reversal in the expected 13A10 binding is observed in the protease treated arms. In the run with the reactants mixed with 5% BSA PBS, an increase in the kallikrein treated arm binding pattern was observed and no difference was seen between the plasmin treated /control arms. This provisional experiment was not one I repeated multiple times. The results however do not support the hypothesis that a plasma co-factor is needed in order for 13A10 to recognise clipped purified β2GPI at the surface of a high binding ELISA plate.

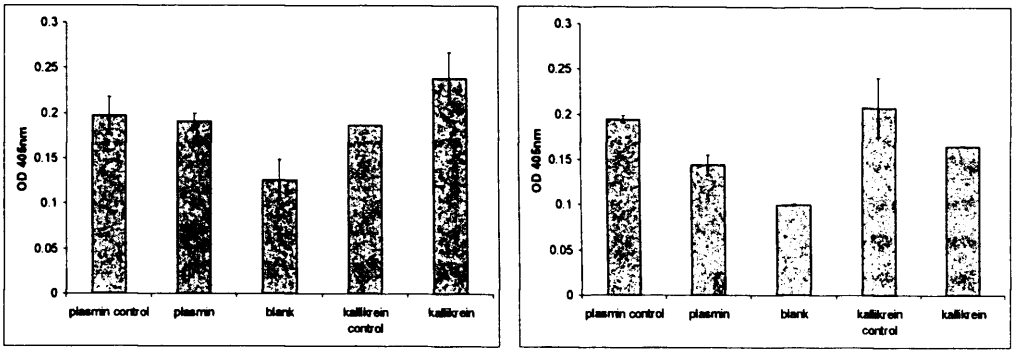


Figure 36 Results of 13A10 binding assay for protease treated pure β2GPI; reactants mixed 1%BSA or pooled normal plasma prior to ELISA plate coating.

Results show the mean and standard deviations of duplicate wells. Blank wells had no 13A10 added in the second layer.

For the immune complex capture assay (number 2) above), a Maxisorp® plate was coated overnight with 200µl/well of a 1 in 1,000 dilution of polyclonal rabbit anti-β2GPI IgG overnight (Dako 09152), as per the β2GPI antigen assay. The plate was then washed three times with PBS and blocked with 200µl/well 1%BSA PBS for one hour.

Control and test (protease treated) reactants prepared overnight as described above were then taken into polypropylene microcentrifuge tubes and incubated for two hours at room temperature as follows: 50µl/reactant; 10µl Aprotinin (10,000 KIU/ml); 50µl 5% BSA PBS; 100 µl 13A10 100µg/ml in 1% BSA PBS.

After washing with PBS, 100µl/well of the immune complex solution prepared above diluted 1 in 10 was added to duplicate wells and incubated for two hours. The plate was then washed three times with PBS, and 100µl/well of a 1 in 1,000 dilution of a goat anti-mouse alkaline phosphatase conjugated antibody (Sigma A2429) (diluted in 1%BS PBS) was added. After washing, the

binding of the anti-mouse antibody was quantified using PNP substrate in diethanolamine buffer as per the anticardiolipin assay. A schematic of this assay is shown in figure 37.

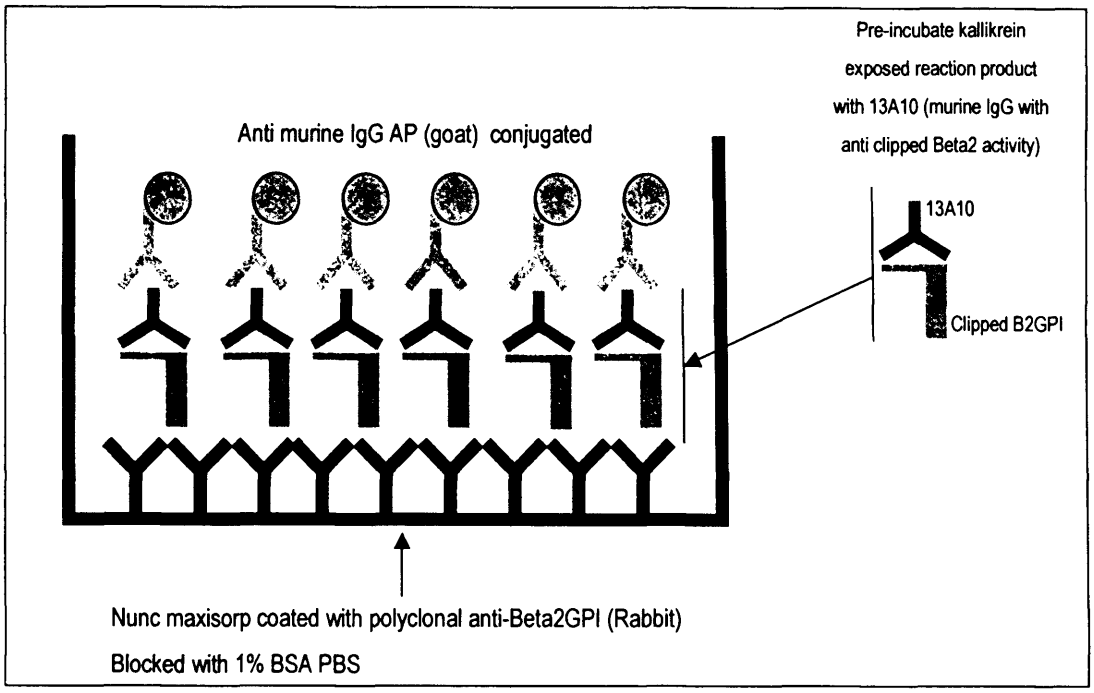


Figure 37 Schematic of the clipped β2GPI-13A10 immune complex capture assay.

The results of this assay showed a consistent increase in the binding of 13A10 to the β2GPI product generated by the action of kallikrein (see figure 38). The results indicate that the binding of 13A10 to purified β2GPI may be influenced by a conformational change occurring at the surface of a high binding ELISA plate. The results also show, that in this system, the binding of 13A10 to clipped β2GPI does not require a plasma co-factor.

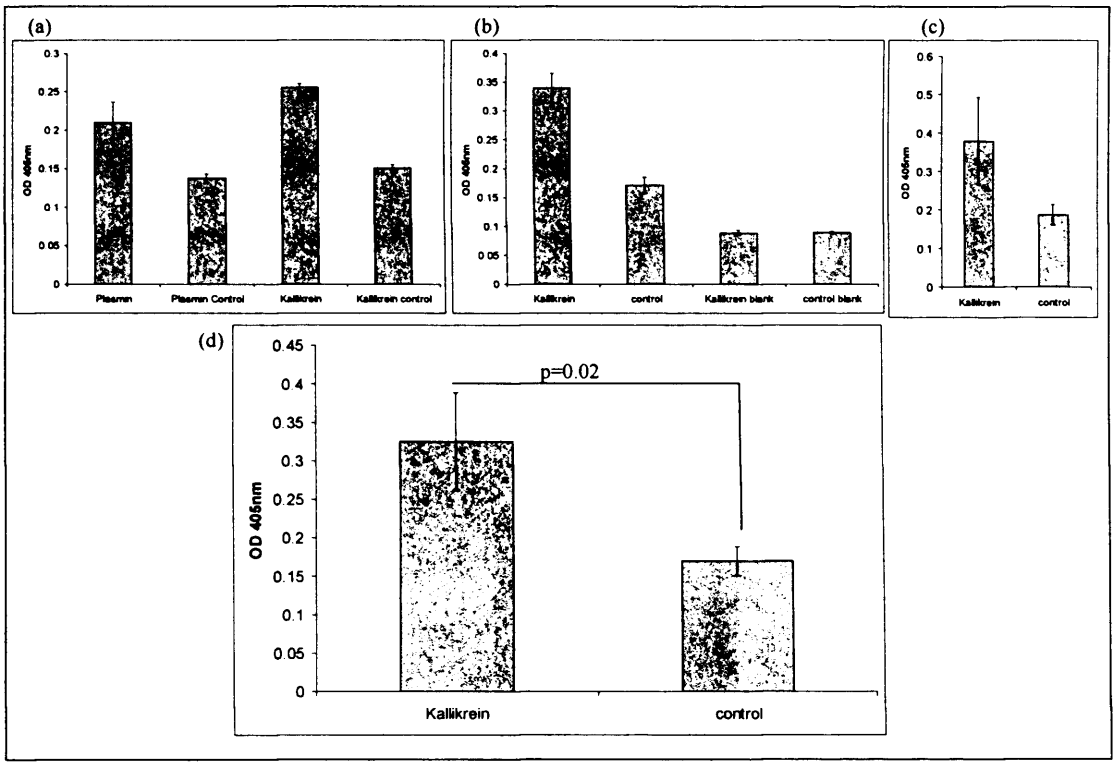


Figure 38 13A10-β2GPI immune complex capture assay results

Assays were carried out as described in the text.
Mean and standard deviation of duplicate wells for plasmin and kallikrein treated β2GPI (a)
Mean and standard deviation of quadruplicate wells for kallikrein treated β2GPI (b) and (c). Blank wells had no 13A10 added in the immune complex step.
Figure (d) shows the mean and standard deviations for the kallikrein treated and control arms of experiments a, b and c. The p value is for a 2-tailed t test.

The method for the 13A10 sandwich ELISA was as follows: wells of a Nunc Maxisorp plate were coated overnight with 13A10 (100μl/well; concentration 10μg/ml in 1% BSA PBS), the plate was then washed three times with PBS and blocked with 1% BSA PBS for 90 minutes. After washing, 50ml/well of aprotinin treated kallikrein exposed and control samples diluted 1 in 10 in PBS were added to wells in duplicate and incubated for 90 minutes. The plate was washed again with PBS and 50μl/well of a 1 in 500 dilution of Horse Radish Peroxidase (HRP) conjugated rabbit anti-β2GPI antibody (Dako PE854) in 1% BSA PBS was added to each well and incubated for 90 minutes. After

washing, binding of the second antibody was detected using TMB substrate as for the β2GPI cardiolipin-binding assay. The results of this experiment are shown in figure 39. Again with a system employing a direct interaction between 13A10 and protease treated pure β2GPI at the surface of a high binding ELISA plate, the signal pattern is the reverse of that one would expect.

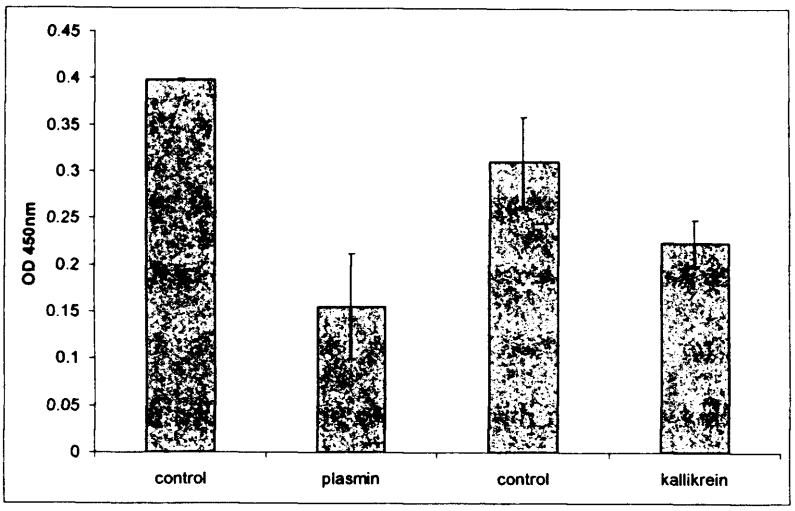


Figure 39 Results of 13A10 clipped β2GPI sandwich ELISA

Results illustrate the mean and standard deviation of duplicate wells for test and control samples.

The results of these three different ELISA set-ups, illustrate that in a non-plasma based system the 13A10 antibody interacts at the surface of a high binding ELISA plate differently to the manner it interacts in a plasma based system. This could be due to the interaction requiring a plasma co-factor or may be due to distortion of the β2GPI molecule when it interacts with the high binding plate resulting in exposure of a cryptic epitope, which can bind 13A10 more avidly than clipped β2GPI. Because the immune complex detection assay produces results indicating 13A10 binding to clipped β2GPI more than non-clipped, it seems likely that a conformational change at the surface of the ELISA

plate is the most likely explanation for the initially paradoxical results seen with pure β2GPI.

Thus, in answer to the question posed at the start of this section, the interaction of β2GPI with kallikrein does produce a product of similar molecular weight change and antigenic structure to that seen on the proteolysis of the molecule by plasmin.

4.3.4 Can aPL interfere with the processing of β2GPI by Kallikrein?

Earlier in this chapter a modest reduction in the cleavage of domain V of β2GPI by plasmin was shown when the reaction was performed in the presence of IgG with aPL activity present. Given this, I investigated whether this phenomenon could also be observed with respect to kallikrein interacting with β2GPI. Because of time limitations, I chose to investigate only two patient samples with regard to this – patient samples 2 and 3 (one of which had and one of which had not shown an effect in the plasmin experiments).

To exclude any significant intrinsic anti-kallikrein activity in the aPL samples, I set up an experiment with a chromogenic substrate specific for kallikrein (MBz-Pro-Phe-Arg-pNA Channel Diagnostics Ltd) as follows: (reactions were performed in triplicate wells on a non-high binding microtitre plate (Alpha Laboratories Ltd)); reagents were kept on ice prior to assay. To each triplicate well was added: 50μl 1% bovine albumin PBS; 50μl kallikrein substrate (final concentration 0.5μM); 50μl IgG fraction from pooled normal plasma or patient 2 and 3 (final concentration 250μg/ml); 50μl kallikrein in 1% BSA PBS (final concentration 1.25μg/ml). The plate was incubated at 37°C and wells read at 405nm every 5 minutes. The results are shown in figure 40, no

apparent difference was seen in hydrolysis of the substrate in the presence of aPL.

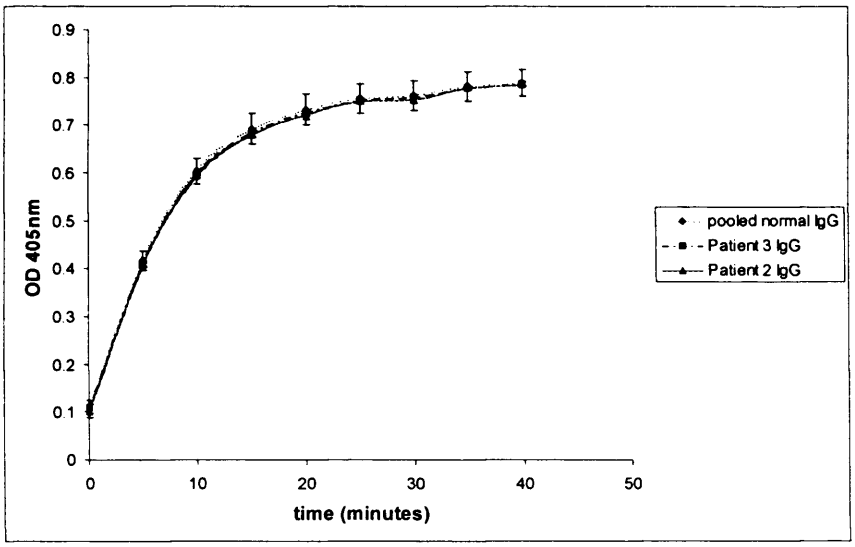


Figure 40 Hydrolysis of chromogenic substrate by kallikrein in the presence of aPL and pooled normal IgG fractions.

To examine β2GPI cleavage by kallikrein in the presence of aPL, I set up triplicate test and control cleavage experiments in a manner similar to that used for the experiments on plasmin described earlier. However, given the fact that the apparent rate of cleavage of β2GPI by kallikrein is much slower than that seen with plasmin, I extended the incubation time to 20 hours. In addition, if I used pooled normal plasma as a source of β2GPI there was a possibility of activating plasmin by kallikrein via uPA. Therefore I used purified β2GPI. (In the course of this work, these experiments were performed prior to the experiments on plasmin and purified β2GPI, which showed no inhibition of cleavage by plasmin in the presence of aPL in contrast to the experiments performed on plasma).

Triplicate test and control tubes for each patient and pooled normal plasma IgG were set up as follows: 100μl IgG fraction in Dulbeco's PBS (final

concentration 0.5mg/ml); 50μl Kallikrein in 1% BSA PBS (final concentration 25μg/ml); 50μl purified β2GPI (Scipac) in 1% BSA PBS (final concentration 25μg/ml). Reagents were kept on ice prior to incubation at 37°C for 20 hours. At the end of the incubation time, 100μl aliquots of test and control samples were taken into 20μl of aprotinin (10,000 KIU/ml) and frozen to -70°C prior to analysis. Samples were assayed in triplicate wells for β2GPI cardiolipin-binding as before at a dilution of 1 in 400 in PBS. The results for triplicate experiments are shown in figure 41.

	Patient 2 IgG	Pooled normal IgG
	0.795	0.842
	0.798	0.825
	0.821	0.843
mean	**0.805**	**0.837**
sd	**0.014**	**0.01**
%cv	**1.74**	**1.19**

p=0.03 independent samples 2 tailed t test

	Patient 3 IgG	Pooled normal IgG
	0.815	0.841
	0.847	0.817
	0.717	0.814
mean	**0.793**	**0.824**
sd	**0.07**	**0.01**
%cv	**8.83**	**1.21**

No significant difference in means

Figure 41 Results for cardiolipin binding ratios on kallikrein treated and control purified β2GPI in the presence and absence of aPL.

The differences in the amount of reduction in cardiolipin-binding in the aPL exposed and non-aPL exposed arms of these experiments was small in contrast to the differences seen in the experiments with plasmin shown earlier. Interestingly if anything there is a trend (and for patient 2 a small but statistically significant difference) towards an increase in kallikrein mediated cleavage in the aPL-exposed arm. This is discussed further in the discussion section.

4.4 An investigation into the interaction of plasmin with domain V polymorphic variants of β2GPI

The interaction of β2GPI and plasmin is clearly (given the site of cleavage) based on the modification of domain V of β2GPI by plasmin. I wanted to see whether this interaction could be influenced by the presence of polymorphic variations in the structure of domain V. During the course of my work on the Cys306Gly polymorphism (Chapter 6) in collaboration with my colleague Dr Ray Camilleri, working on the Trp316Ser polymorphism of β2GPI, two patients emerged with domain V polymorphic changes that could help to answer this question. The molecular biology methods used to detect the Cys306Gly β2GPI polymorphism discussed in this section are described in Chapter 6 of this thesis along with a discussion of genetic polymorphisms in β2GPI.

4.4.1 Genetic screening for the TrpSer316 polymorphism

Genetic screening for the Trp316Ser polymorphism was carried out by a colleague in our department. Full details of the method used can now be found in the literature (Camilleri *et al*, 2003) (Nash *et al*, 2003). Briefly, the 316 polymorphism was detected by amplifying exon 8 of the β2GPI gene using PCR and forward primer 5'TAACAAATGATTGTTTCTCTTAGAATG3' and reverse primer 5'AATGACATAACTAAAAGTAAGCTACC3'. PCR regime as follows: 95°C/5min (1 cycle); 95°C/30sec; 60°C/30sec; 72°C/5mins (1 cycle). The PCR product was digested with *Sfu*I to distinguish the Trp (TGG) and Ser (TCG) codons.

4.4.2 A discussion of the patients whose samples were analysed in this section

Genetic screening of a large patient cohort revealed two patients who could help with an investigation into the effects of domain V polymorphisms on the interaction of plasmin with β2GPI. One was homozygous for the Trp316Ser polymorphism and the other a compound heterozygote for the Trp316Ser and Cys306Gly polymorphisms.

The patient who was homozygous for the Trp316Ser polymorphism raised some interesting questions about the pathophysiology of APS and was the subject of a published case report prepared during this thesis work (Nash *et al*, 2003). An outline of this patient's case is shown in table 19.

Case History

The patient is a woman with a past obstetric history of a termination of pregnancy aged 21, a stillbirth (28 weeks) with intrauterine growth restriction and hypertension aged 30 and a miscarriage (9 weeks) in the same year. She has had three successful pregnancies aged 31, 33 and 35 years, when managed antenatally with aspirin and prophylactic low dose low molecular weight heparin (LMWH). She has no personal history of arterial or venous thrombosis. Her past medical history includes essential hypertension diagnosed in 1992. Her present drug history consists of Atenolol and the progestogen only pill.

The patient has demonstrated a positive titre for IgG anticardiolipin antibodies since she was investigated following her stillbirth (lupus anticoagulant negative). She has also demonstrated positivity for IgG anti-β2GPI antibodies over a two-year period (see below). Other thrombophilia tests (antithrombin, protein C, protein S, modified activated protein C resistance, factor V Leiden and prothrombin G20210A polymorphism) were unremarkable, as was the patient's blood count and routine biochemistry.

The patient was found to be homozygous for the thermolabile variant of methylene tetrahydrofolate reductase. Further investigation revealed the patient to be positive for IgG anti-factor XII antibodies but negative for anti-tissue factor pathway inhibitor antibodies.

Date	IgG aCL (GPLU)	IgM aCL (MPLU)
	(normal <5 GPLU)	(normal<5 MPLU)
Jul' 1996	14.6	0
Sept' 1996	4.4	0
Nov' 1996	18.8	2.6
Dec' 1996	10.1	0
Feb' 1997	17.8	0.8
Oct' 1997	16.6	0
Jun' 1998	15.6	0.5
Nov' 1998	20.8	3.1
May' 1999	15.3	0.1
Nov' 2001	13.8	0

Date	IgG anti-Beta2GPI (%)
	(normal <3.5%)
June '2000	39
Dec' 2000	29
May' 2001	28

Table 19 Case history of the patient found to be homozygous for the β2GPI 316 polymorphism

The case of this patient was interesting, since it illustrated someone with obstetric pathology related to aPL positivity, which is clearly modified by treatment with aspirin and LMWH, but in whom the endogenous β2GPI is

unable to bind to phospholipid. In a survey of the literature, I could find no patients described who are Ser316Ser homozygous for the β2GPI Trp316Ser polymorphism that also have anti-β2GPI antibodies. A patient heterozygous for β2GPI polymorphisms at 316 and 306 with APS has been described (Gushiken *et al*, 2000). Given the fact that this patient's β2GPI fails to bind phospholipid, it is interesting that she possesses persistent anti-β2GPI activity that appears to be pathogenic in relation to pregnancy.

One explanation for this could be that another aPL cofactor protein is important in this patient's condition. Some authors have suggested that anti-β2GPI antibodies have only a limited role in the pathogenesis of recurrent pregnancy loss and that anti-annexin V activity may be of more importance. In addition there are reports that aPL can reduce annexin V on the surface of trophoblastic cells (Rand *et al*, 1997a;Rand *et al*, 1997b).

IgG aCL alone are associated with adverse pregnancy outcomes (Alfirevic *et al*, 2002). It is therefore possible that the patient's positive aCL titre is important with regard to her pregnancy loss. Given however the fact that her aCL titre is only moderately raised, it is likely that much of this aCL titre contains anti-β2GPI activity. This patient does however also exhibit a positive titre for IgG anti-factor XII antibodies which have been shown to be associated with recurrent fetal loss (Jones *et al*, 2001).

However this case still presents us with a patient who has a non phospholipid-binding variant of β2GPI who has a positive IgG anti-β2GPI titre. The placenta expresses paternal derived antigens. Moreover, expression of β2GPI has been demonstrated in the placenta from as early as eleven weeks gestation (Donohoe *et al*, 2000). Thus, the possibility arises that the patient may

have mounted an alloimmune response against paternal β2GPI expressed on the placenta resulting in her antenatal manifestations of APS and that this has been modified by therapy with aspirin and low molecular weight heparin.

The patient with compound heterozygosity for the 306 and 316 polymorphisms, had presented with a cerebral venous thrombosis but had manifested only transient aCL and LA activity (probably related to her cerebral event).

An example of a cardiolipin-binding curve for the patient with compound heterozygosity for the 306 and 316 polymorphisms is shown in figure 42.

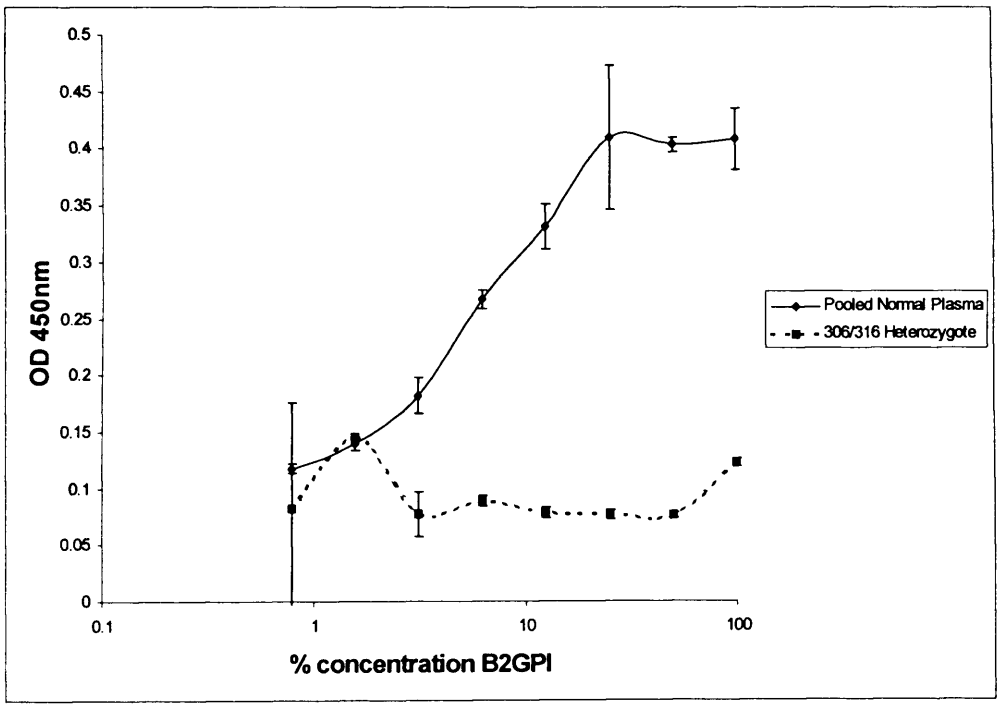


Figure 42 β2GPI-Cardiolipin-binding curve for a patient with compound heterozygosity for the 306 and 316 polymorphisms.

The graph shows the mean and standard deviation of duplicate wells in the ELISA assay. Samples from the pooled plasma and patient were normalised to each other so β2GPI concentrations were equal at each dilution. 100%=pooled normal plasma at 1 in 200 dilution. A similar curve is observed for the patient homozygous for the 316 polymorphism.

4.4.3 Preliminary experiments on the effect of plasmin on domain V β2GPI variants

In a preliminary experiment, I examined the effect of plasmin on pure β2GPI, β2GPI from a subject with a wild-type β2GPI genotype and the patient who was homozygous for the Trp316Ser polymorphism. Plasma samples were acetone treated and 100μl of acetone treated plasma mixed with 300μl of plasmin (100μg/ml in 1%BSA PBS); purified β2GPI (Scipac) (200μg/ml in 1% BSA PBS) was mixed in the same proportions as the plasma with plasmin. The samples were incubated at 37°C for 240 minutes before the reaction was stopped by adding aprotinin (as before) and freezing to -70°C prior to analysis. Because the Trp316Ser variant of β2GPI does not bind cardiolipin, I could not use the cardiolipin-binding assay to assess cleavage of domain V. I therefore employed SDS-PAGE and western blotting (as described earlier) to detect the molecular weight change in β2GPI, which corresponds to cleavage of β2GPI by plasmin. The resulting gel is shown in figure 43.

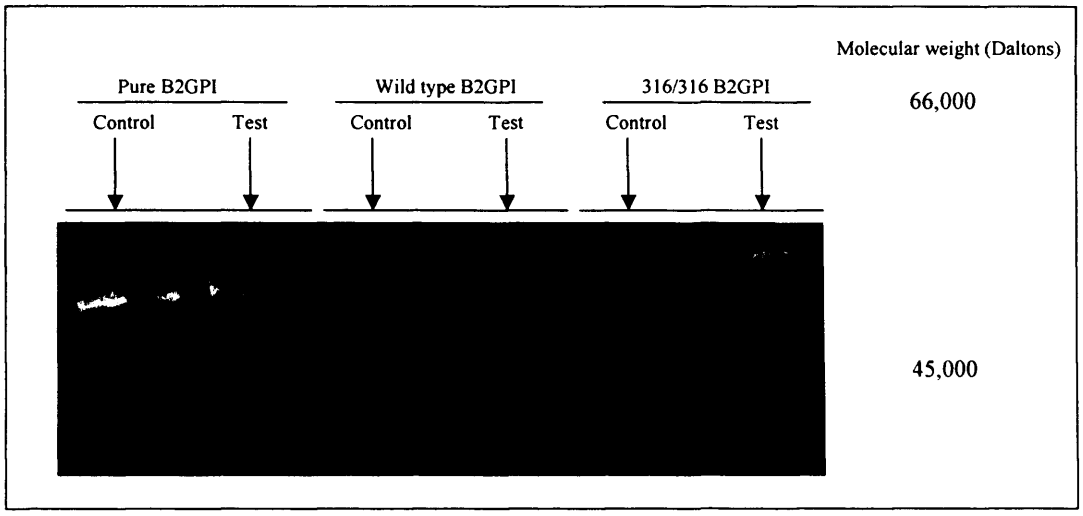


Figure 43 SDS-PAGE and western blotting comparing normal β2GPI and β2GPI with the Trp316Ser polymorphism after 240 minutes of plasmin treatment.

4.4.4 Analysis of clipping with normalised β2GPI levels and removal of IgG fraction

While the preceding experiment indicated that the 316 polymorphism of β2GPI was resistant to cleavage by plasmin, the experiment was flawed at several levels. These included a failure to normalise β2GPI concentrations between plasma samples and a failure to remove anti-β2GPI activity from the 316/316 patient plasma (since this activity had been previously shown to reduce plasmin mediated cleavage of β2GPI). In order to address this I repeated the work (this time also with the compound heterozygote for the 306 and 316 polymorphisms) but ensured that the β2GPI concentration of the samples were normalised, that there was no anti-β2GPI activity present in the sample and performed the experiment over a longer time course.

To deplete the samples of IgG anti-β2GPI activity, after acetone treatment, patient and pooled normal plasma samples were diluted 1 in 3 in PBS and passed three times over a protein G sepharose column. Between each passage the column was washed as per the protocol for purification of IgG. The dilute samples were then "re-concentrated" using a Centricon® TM30 micro-concentrator (molecular weight cut off 3,000 Daltons). An absence of residual anti-β2GPI activity was confirmed using the anti-β2GPI assay as described in the general methods section. The concentration of β2GPI in each sample was determined using the β2GPI antigen assay described in the general methods section. Plasmin cleavage was carried out on samples normalised by dilution in PBS for β2GPI antigen level (final concentration β2GPI 25μg/ml) with a final plasmin concentration of 50μg/ml at 37°C. Sub-aliquots were taken into aprotinin (as described previously) and frozen immediately to -70°C at 0, 1, 2, 3, 4, 5, and 22 hours. The aliquots were then run on SDS-PAGE with western blotting for β2GPI as described previously (on 8-16% gradient BioRad® 345-0037). The results of this time course experiment are shown in figure 44.

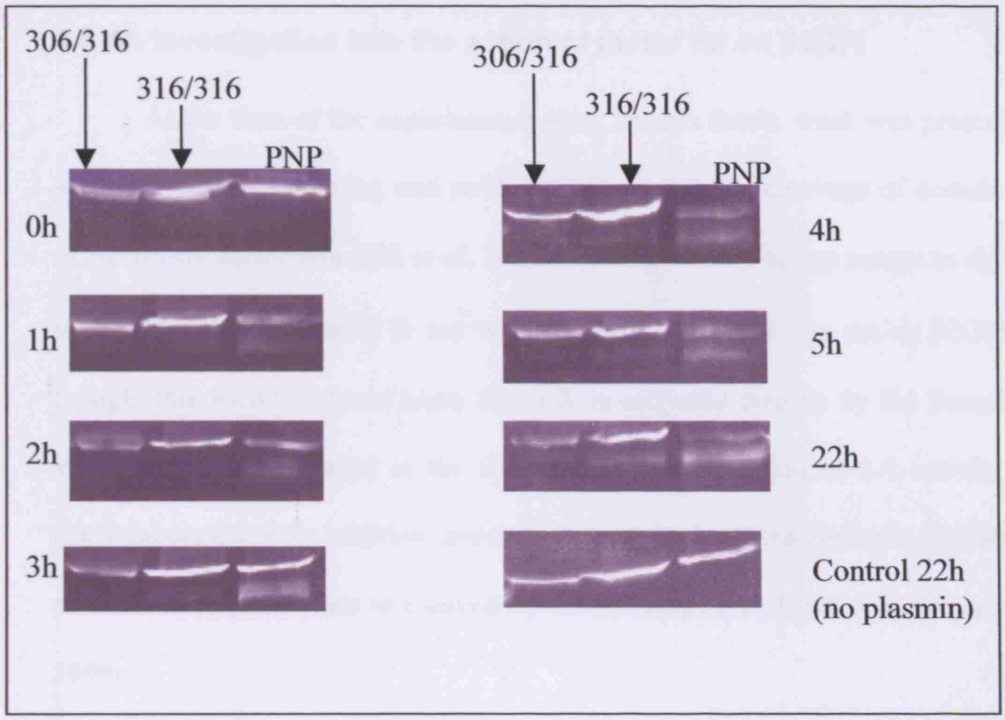


Figure 44 SDS-PAGE and western blotting of plasmin treated β2GPI from pooled normal plasma (PNP), a patient homozygous for the 316 β2GPI polymorphism and a patient with 316/306 compound heterozygosity for β2GPI polymorphisms.

The results illustrated above, show that there is reduced cleavage (as determined by the presence of a molecular weight change in β2GPI) by plasmin in the samples with domain V β2GPI polymorphic variants. At 3 hours there is a second band appearing in the 316/316 arm however this band is much less intense compared to that in the PNP arm. At 22 hours, a band is visible in the 316/316 arm (and faintly in the 316/306 arm) but again much less intense compared to the PNP second band.

4.5 An investigation into the action of factor Xa on β2GPI

At the time of the experimental work on this thesis, work was presented at an international meeting and published illustrating the cleavage of domain V of β2GPI by factor XIa (Shi *et al*, 2005). Having already set up assays to detect such processing, I decided to see whether factor Xa could also act on β2GPI. I thought this was of interest since factor X is activated directly by the Russell's viper venom (RVV) used in the DRVVT test for detection of LA activity in many laboratories. In addition, previous reports in the literature have conflicted as to whether β2GPI can be cleaved by Xa (Ohkura *et al*, 1998) (Horbach *et al*, 1999).

To screen for Xa activity on β2GPI, I set up a simple experiment in triplicate with test and control (no Xa) arms incubated for 20 hours. Reactions were carried out at 37°C in 1% bovine albumin TRIS buffered saline (TBS); final concentrations of reagents: β2GPI (Scipac) 100μg/ml; Human Xa 100μg/ml (Enzyme Research 2530PAL). (TBS: pH 7.8; TRIS 50mM; NaCl 12mM). Samples were frozen after Xa treatment to -70°C prior to assay for β2GPI cardiolipin binding. The results of the cardiolipin binding for Xa treated β2GPI is shown in figure 45.

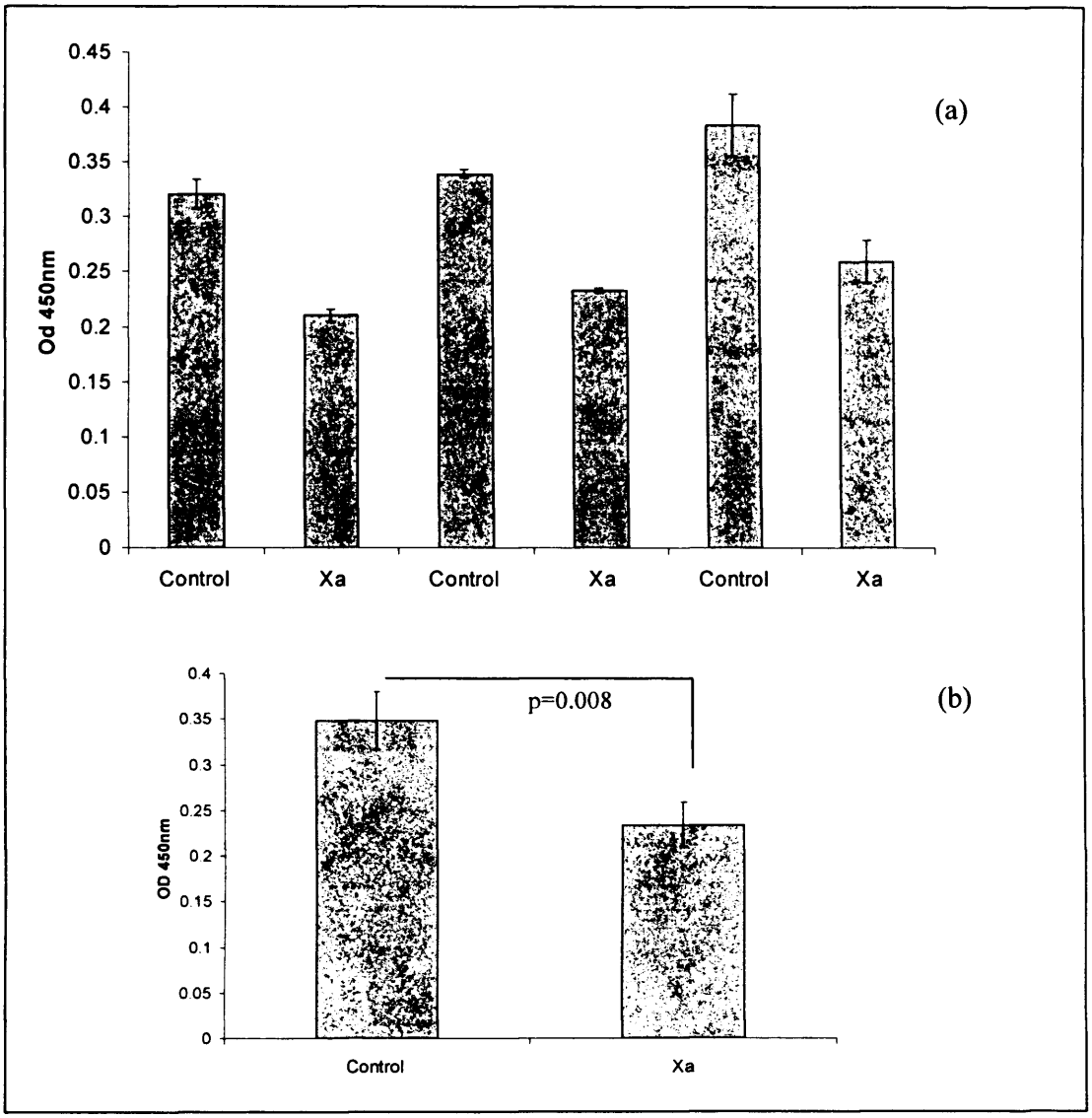


Figure 45 Cardiolipin binding results for Xa treated and control β2GPI

The results of three separate experiments (a) and their combined means and standard deviations (b) are shown. Results in (a) are the mean and standard deviations of triplicate wells in the binding assay. The p value is for a 2-tailed t-test.

These results showed a clear reduction in the binding of β2GPI to cardiolipin following treatment with Xa. As with the kallikrein experiments discussed earlier, this experiment did not exclude the possibility that the Xa was competing with the β2GPI for phospholipid binding sites in the cardiolipin-binding assay. To exclude this possibility, I repeated the experiment this time

however, an arm was added with benzamidine (0.3M) to inhibit the action of Xa. (This concentration had previously been shown to inhibit Xa at the concentration used in these experiments using a chromogenic substrate assay). The results of this experiment are shown in figure 46.

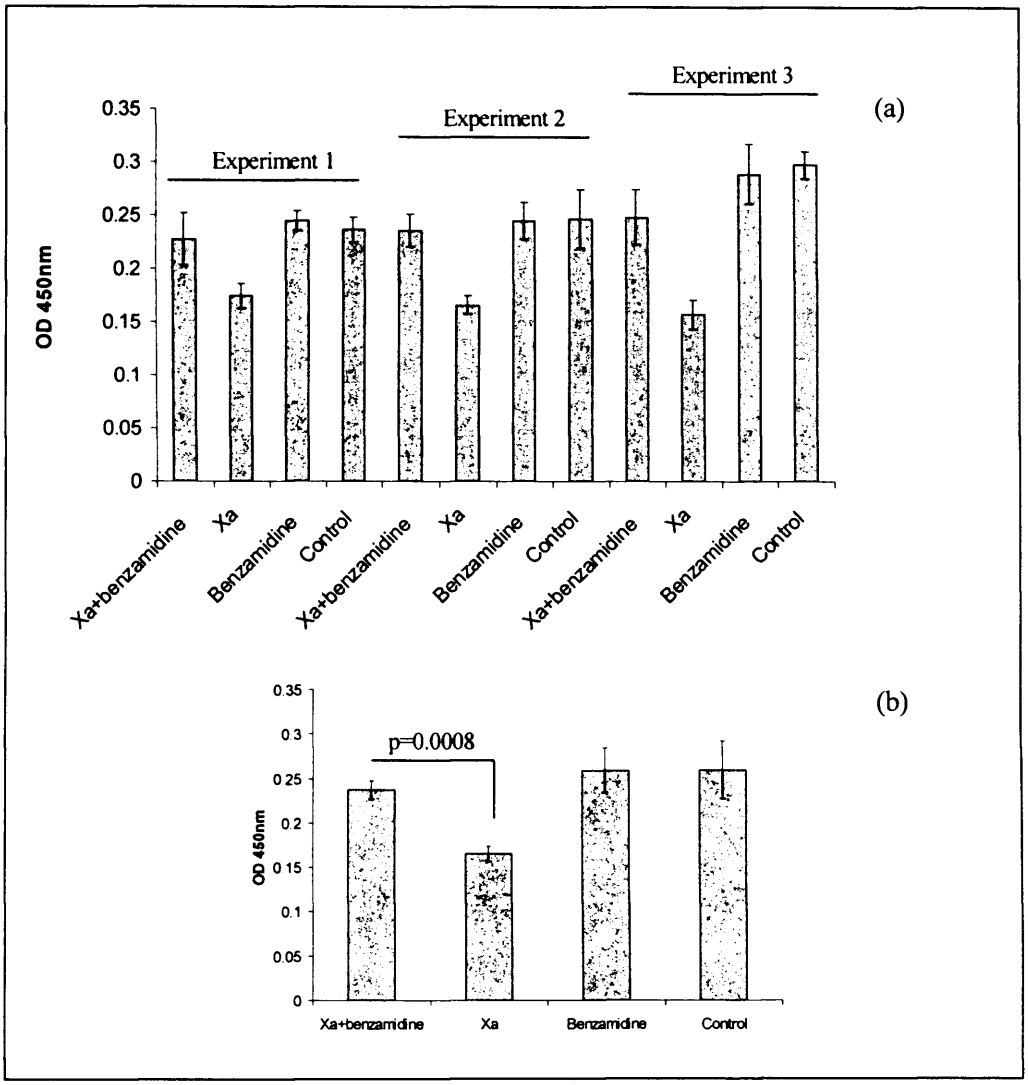


Figure 46 Cardiolipin binding results for Xa treated and control β2GPI with and without the presence of 0.3M benzamidine.

(a) The results of 3 separate experiments. (b) Amalgamated results showing a significant difference in binding for Xa treated β2GPI with and without the presence of Xa inhibition by benzamidine. (The benzamidine arm consisted of purified β2GPI and benzamidine only; the control arm β2GPI only).

These results confirmed that the reduction in β2GPI binding observed on incubation with Xa was likely to be due to some action of Xa on the β2GPI

molecule rather than competitive inhibition of binding at the phospholipid surface.

4.6 Discussion

4.6.1 The effect of aPL and β2GPI polymorphisms on the plasmin mediated cleavage of β2GPI.

In the first section of this chapter, I have presented results, which indicate that some aPL containing IgG fractions can cause a small reduction in the observed cleavage of domain V of β2GPI treated with plasmin. This effect was observed for three of the patient IgG samples with an observed high level of anti-β2GPI activity. The magnitude of this reduction in cleavage is modest being around 10% when cardiolipin-binding ratios are compared between patient and pooled normal plasma IgG results. The effect was not seen in a patient with low titre aPL or in a patient with aPL secondary to leprosy. The effect was also not seen when the experiment was repeated in a plasma-free system using purified β2GPI.

Criticisms of this work must include the limited number of patient samples examined. I deliberately focused on IgG fractions which I knew possessed a high titre of anti-β2GPI activity for this work as I felt these samples would be most likely to provide insight into whether the effect observed in the initial patient samples could be seen in others. Future work however should focus on looking for this effect in patients with lower aPL titres and perhaps in those without anti-β2GPI activity. That the reduction in plasmin cleavage was seen with 3 out of the six aPL samples examined is I feel a result of my bias in choosing samples to assay. I would be surprised if this effect was observed in 50% of APS patients. (It is worth noting however that a non-significant trend towards inhibition was seen with the other three IgG patient fractions). In

addition, this work compared the patient IgG samples to a single pooled normal plasma IgG fraction. A better approach may have been to run several non-pooled aPL negative IgG fractions to serve as a control population. Another aspect of experimental work for the future would be to run the aPL inhibition experiments over a time course rather than a snapshot experiment as presented here.

Given the observation that half of the IgG samples used showed no inhibition of plasmin in a chromogenic assay (and those which did showed very little inhibition), an explanation other than direct plasmin inhibition is needed to explain all of these findings. (A recent paper looking at anti-plasmin activity in APS also failed to demonstrate significant inhibition of plasmin acting on a chromogenic substrate (Yang *et al*, 2004); they did however point out that small chromogenic substrate molecules may not reflect the enzymic action on larger molecules in the presence if inhibitory aPL). Moreover, given that it is likely that most aPL bind β2GPI outside epitopes on domain V (Giles *et al*, 2003b), it seems unlikely that the effect is due to direct binding of aPL to the Lys317-Thr318 cleavage site. That IgG with anti-β2GPI activity can inhibit cleavage of β2GPI by plasmin has been demonstrated for the murine monoclonal antibody Cof-18 (Matsuura *et al*, 2000). However this antibody was directed against specific epitopes on domain V of β2GPI, it is also a monoclonal antibody as opposed to the polyclonal anti-β2GPI activity likely to reside in the patient IgG fractions. This paper does however give precedent for antibody activity being able to impede the action of plasmin on β2GPI. Moreover, one could speculate as to whether the patient samples showing an effect on plasmin cleavage of β2GPI possess anti-domain V activity not seen in the other samples.

One possibility, which could explain these findings independent of a direct action on domain V, is that the binding of aPL to β2GPI creates steric hindrance to the interaction of plasmin with the molecule. Another mechanism could be that by dimerising β2GPI, the antibodies bring adjacent domain V regions into close proximity and this has a protective effect. Moreover, the dimerisation of β2GPI is known to increase the affinity of the molecule for phospholipid (Takeya *et al*, 1997) and this mechanism could again reduce the ability of plasmin to interact with the protein, specifically at the cleavage site in domain V.

This latter mechanism could explain the lack of effect on plasmin cleavage seen in the experiments with purified β2GPI. It is possible that residual phospholipid in the pooled plasma, not present in the purified β2GPI experiments could contribute to this effect. Alternatively, another component of plasma (again not present in the purified β2GPI experiments) could be interacting to perhaps increase the interaction of the aPL with the β2GPI molecule. The amount of cleavage noted in the purified-β2GPI experiments was however slightly greater than that seen in the experiments which used pooled plasma as a source of β2GPI. It is therefore possible that these experiments had been allowed to run beyond the point at which any difference in cleavage between the aPL and control samples could be discerned. Another difference between the experiments with purified β2GPI and the plasma-based experiments is that the system in the plasma-based experiments was treated with acetone.

The findings in this section could be said to be at variance with the paper by Itoh *et al*, which described the presence of increased cleaved β2GPI in patients with LA activity (Itoh *et al*, 2000). This paper however did not find

evidence for increased fibrinolytic activity in the LA positive group (in contrast to the leukaemic patients studied), and discusses the possibility of another protease being responsible for the increased β2GPI cleavage observed. Given the fact that XIa (Shi *et al*, 2005), Xa (Ohkura *et al*, 1998) and kallikrein (see this thesis) can cleave β2GPI it is entirely possible that another protease could be contributing to this finding. In addition, my work does not show a complete absence of plasmin mediated cleavage in the presence of aPL and if anything, the difference seen in the presence and absence of aPL is small.

One area where the small difference noted in the amount of β2GPI cleaved by plasmin could be of importance relates to the observations of Yasuda *et al* on the presence of cleaved β2GPI as a marker for fibrinolysis in acute stroke and lacunar infarcts (Yasuda *et al*, 2004). It may be possible that the inhibition of plasmin cleavage of β2GPI by aPL could affect the amount of cleaved product detected in an aPL positive patient – the paper does not mention how many of the cerebral ischaemia patients in the study were persistently positive for aPL.

If aPL are able to reduce the plasmin mediated cleavage of β2GPI, another effect of this may be that they are able to locally maintain a population of β2GPI, which is still able to interact with cell surfaces when fibrinolytic potential is increased. Given that β2GPI is a likely player in the cell activation events seen on aPL exposure to endothelial cells this could provide a mechanism whereby aPL are able to potentate their own activity by protecting β2GPI from cleavage in domain V. Moreover, clipped β2GPI may be cleared from the circulation more rapidly than the intact form (Horbach *et al*, 1999). A reduction in cleavage of β2GPI may therefore reduce clearance from the plasma of β2GPI.

Some tautologies exist however when one considers the actions of clipped β2GPI in relation to its direct actions on the coagulation and fibrinolytic mechanisms. Clipped β2GPI is known to impede extrinsic fibrinolysis (Yasuda *et al*, 2004), therefore one could postulate that by decreasing the formation of clipped β2GPI, that aPL would have a total pro-fibrinolytic effect. On the other hand, clipped β2GPI looses its potential to inhibit XI activation. Although XIa is procoagulant, its activation will also (via thrombin generation) lead to increased TAFI activation and therefore reduced fibrinolysis. In all, local factors (such as the expression of thrombomodulin) are likely to determine which of these factors prevails at any one time. One could also debate the location of plasmin cleavage of β2GPI. Ohkura *et al* discussed whether the cleavage of β2GPI tended to occur in a milieu protected from inhibitory serpin (α2PI) activity such as the fibrin clot or perhaps in the extracellular matrix. The work of Horbach *et al* does however show that under certain circumstances (sepsis, DIC and fibrinolytic drug treatment) that significant levels of clipped β2GPI are detectable in the plasma.

Plasmin has been shown to be capable of degrading IgG (Chuba, 1994) (Harpel *et al*, 1989). One therefore must be mindful of the possibility that in areas of local high fibrinolytic potential, that plasmin itself could be active against any aPL present itself.

That β2GPI with amino acid changes at positions 306 and 316 have reduced interactions with and reduced proteolysis by plasmin, is perhaps not surprising given the large structural changes these polymorphisms bring about in domain V. Given the interactions of clipped β2GPI and glu-plasminogen described by Yasuda *et al* it would be interesting to find out whether these

variant proteins have any inhibitory effects on fibrinolysis in a manner similar to that observed for the clipped β2GPI which also has a disrupted domain V.

4.6.2 The proteolysis of domain V of β2GPI by plasma kallikrein (and Xa)

The work presented in the second section of this chapter shows that kallikrein can act on β2GPI and cleave domain V. This was demonstrated by means of reduction in binding of kallikrein-exposed β2GPI to cardiolipin, SDS PAGE and using an antibody specific for plasmin cleaved β2GPI. That this activity could take place in a purified protein system indicates that this action of kallikrein is independent of any indirect action of kallikrein on β2GPI mediated by it activating plasminogen (either directly or via uPA activation). Moreover, the kallikrein inhibitor DX-88 was shown to inhibit the action of kallikrein on β2GPI. (The DX-88 experiments also indicate that the effect of kallikrein on the binding of β2GPI to phospholipid is not due to kallikrein competing for cardiolipin binding sites with β2GPI).

The duration of incubation of kallikrein and β2GPI used in most of the experiments presented here was long for a physiological process. It is possible that more efficient reaction kinetics can occur physiologically via the interaction of membrane bound receptors for kallikrein and locally mediated high concentrations of proteolytic enzyme. The time course experiments do however show some decrease in cardiolipin binding of β2GPI after two hours of kallikrein exposure.

As mentioned earlier, kallikrein activation is known to have pro fibrinolytic effects mediated via uPA activation and via the actions of bradykinin. Given the anti-fibrinolytic effects of clipped β2GPI, cleavage of β2GPI may provide a pathway of negative feedback on the pro-fibrinolytic activities of

kallikrein. Previous work has identified members of the contact system as targets for aPL activity (Sugi & McIntyre, 2001) (Sugi & Makino, 2000). However, in a limited experiment I could find no evidence of aPL being able to significantly impede the process of kallikrein cleavage of β2GPI. There was in fact a small (and in one case significant) increase in cleavage in the aPL exposed samples.

One area within my work on the action of kallikrein on β2GPI was my attempt to use the monoclonal antibody 13A10 to detect the clipped form of β2GPI in a purified protein system. As shown in the relevant results section, in a plasma based system, 13A10 readily detects clipped β2GPI post treatment with kallikrein (see figure 34) in an ELISA system utilising coating of the reaction product to the surface of a high binding ELISA plate. The only paper in the literature describing the characteristics of 13A10 is that of Horbach *el al.* In this work they describe two clipped forms of β2GPI formed on cleavage of β2GPI in domain V which they designate β2GPI*1 and *2. 13A10 is only able to recognise the *1 form. However the two forms have identical N-terminal sequences. Interestingly, in their paper, Horbach *et al* describe being unable to detect the presence of clipped β2GPI at the surface of a high binding ELISA plate when β2GPI is clipped at the plate surface. This is in contrast to their ability to detect the clipped protein in purified form (post affinity chromatography and isolation of the relevant peak) at the surface of a high binding ELISA plate. This raises the possibility that 13A10 may have problems with detection of clipped β2GPI in the presence of a protease with affinity for domain V of β2GPI (in their case plasmin). The results of my work illustrate that in some way (be it interaction with another protein at the plate surface or a conformational change in β2GPI when bound to a high binding ELISA plate) 13A10 gives a "reverse signal"

when the clipped protein is directly bound to the ELISA plate using purified β2GPI. This effect was not fully abrogated by the presence of plasma or BSA. However when an immune complex capture assay was used one could detect clipped β2GPI (as a result of the action of plasmin or kallikrein) as expected. My results indicate that the most likely reason for this relates to the conformation of purified β2GPI (both clipped and non-clipped) when bound to a high binding ELISA plate. This effect may be magnified by the presence of a protein, which can interact with domain V. A plasma factor appears to be able to abrogate this effect when a whole plasma system is used for the cleavage stage of the experiment. Changes in the tertiary structure of β2GPI resulting in the exposure of cryptic epitopes in the protein upon its interaction with an ELISA plate or negatively charge phospholipid are described in the literature (Pengo *et al*, 1995) (Kuwana *et al*, 2005) (Igarashi *et al*, 1996) (Matsuura *et al*, 1994). In this case, purified β2GPI appears to take on a conformation at the surface of a high binding ELISA plate, which opens up an epitope in intact β2GPI for 13A10 and in some way, obscures the 13A10 epitope on clipped β2GPI. This conformational change is not seen in the liquid phase where 13A10 binding to clipped β2GPI is greater than that seen for the intact protein (as reflected by the results of the immune complex capture assay).

The experiments performed to examine the effect of factor Xa on β2GPI show a reduction in binding of β2GPI to cardiolipin post-treatment with factor Xa. Moreover, the inhibition experiments with benzamidine reveal this effect to be unlikely to be due to a competitive effect by factor Xa at the cardiolipin surface. These results are at variance with those of Horbach *et al* but in concordance with those of Ohkura *et al*. The experiments in the Horbach paper

involved the attempted proteolysis of β2GPI by factor Xa being performed at the surface of a high binding ELISA plate, while the work in the Ohkura paper was carried out in liquid phase (albeit with domain V recombinant fragments rather than intact β2GPI). Perhaps factor Xa is unable to interact with β2GPI at the surface of an ELISA plate (unlike plasmin, trypsin and elastase) resulting in these discrepant results. The ability of factor Xa to cleave β2GPI provides another level for reduction in β2GPI inhibition of XI activation on activation of the clotting mechanism. Moreover, given that Russell's viper venom is a direct X activator, it seems likely that in the DRVVT assay, a quantity of β2GPI with reduced phospholipid binging capacity may be generated.

A diagram summarising the findings of this thesis in relation to other work in the literature on the cleavage of domain V of β2GPI is shown in figure 47.

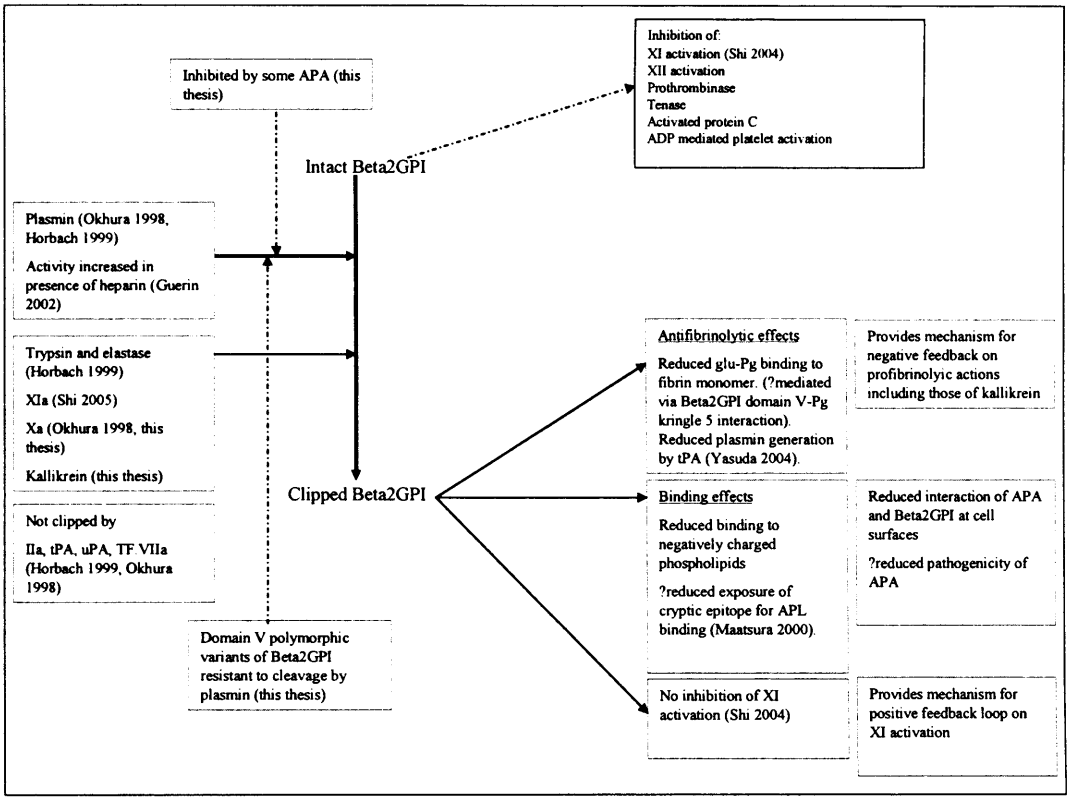


Figure 47 Summary of the enzymes leading to, the effects of and factors influencing the cleavage of β2GPI.

Chapter 5

An investigation of the interaction of antiphospholipid antibodies with components of the fibrinolytic system

5.1 Introduction

As discussed in the introductory chapters to this thesis, many mechanisms have been proposed as to the pathogenic mechanisms exerted by aPL. Impairment of the fibrinolytic mechanism has been suggested by some authors as one such mechanism. Such an effect could help to explain the "two-hit" phenomenon seen in some APS patients, whereby an initial prothrombotic stimulus such as inflammation is followed by a thrombotic event.

Within the work contained in this section I sought to examine whether aPL could influence the interaction of components of the fibrinolytic system (particularly plasminogen) at the surface of endothelial cells. As discussed earlier, this interaction forms an important mechanism for amplification of the fibrinolytic system and given that aPL are known to interact with endothelial cell surfaces, was a candidate for a site of interaction between the two systems. At the time the experimental work was performed, no work had appeared in the literature examining this interaction. Additionally, I wanted to test whether using the aPL fractions form the patients in our cohort, I could reproduce the phenomenon of delayed clot lysis times in the presence of aPL which has previously been described.

5.1.1 Background

There are many theories as to why patients with APS develop thrombotic and obstetric complications at an increased rate compared to the general population. With regard to fibrinolytic impairment being important in APS it is interesting to remember the concept of a "two hit" process in APS; perhaps any impairment of fibrinolysis by aPL will prolong the presence of a clot which may have formed due to another predisposing factor such as infection or inflammation. This section will review the literature with regard to whether an interaction between APS and fibrinolysis could lead to some of the manifestations of APS.

An early study (Keeling *et al*, 1991) which examined the levels of PAI and tPA in patients with SLE and APS found that while both groups exhibited raised levels of both proteins, in the APS group the fibrinolytic response to venous occlusion was decreased due to increased levels of PAI. A similar result was found by a later study (Jurado *et al*, 1992) which examined the fibrinolytic response in patients with connective tissue disease. The study concluded that an impairment of fibrinolysis (measured by euglobulin fibrinolytic activity on fibrin plates) was seen in patients with connective tissue disease mainly due to raised PAI I levels, however, these changes were not found to correlate with either the incidence of thrombosis or the presence of aPL in this cohort.

5.1.2 Autoantibody activity against components of the fibrinolytic system

As well as looking for systemic changes in fibrinolysis, other work has concentrated on whether autoantibody activity directed against components of the fibrinolytic system can be detected in patients with APS. Autoantibody activity against epitopes on human tPA have been demonstrated in patients with

APS (Cugno *et al*, 2000) (Cugno *et al*, 2004). In the latter study, some of these anti-tPA antibodies exhibited activity against the catalytic domain of tPA. However, there was no correlation between aCL titre and anti-tPA activity suggesting that the antibodies represent two separate populations. Moreover, the antibody subclasses were different (IgG1 and 3 for anti-tPA and IgG2 for aCL).

Plasminogen may also represent a target for autoantibodies. Some patients with APS manifest anti-prothrombin antibodies and a study in patients with myocardial infarction has suggested that anti-prothrombin activity and anti-plasminogen activity may overlap (Puurunen *et al*, 1998). The same group also found that after immunisation of mice with either human prothrombin or plasminogen, that cross reactive antibodies are a frequent finding (Puurunen *et al*, 2001). There are common motifs to plasminogen and prothrombin most notably their kringle domains. Indeed it has been demonstrated that monoclonal antibodies directed against kringle 2 of prothrombin also recognise the kringle 5 domain of plasminogen (Church & Messier, 1991). A later study (Simmelink *et al*, 2003) showed that while anti-prothrombin and anti-plasminogen activity can be detected in patients with APS and SLE, there was no correlation between the two activities. The authors of this paper also concluded that while anti-prothrombin activity was associated with thrombosis, anti-plasminogen activity was not. Anti-prothrombin antibodies were associated with lupus anticoagulant activity unlike anti-plasminogen antibodies. The studies by Puurunen and Simmelink did however look at quite different patient groups. The former examined a cohort of patients on whom prospective samples has been taken in a study of ischaemic heart disease, while the latter looked at patients with a thrombotic history, the majority of whom had SLE.

Plasminogen binds to cell surfaces and fibrin, and the ability of IgG from the sera of patients to bind to plasminogen in a bound state has been investigated (Dominguez *et al*, 2001). This study found antibody activity against bound plasminogen in approximately 10% of the APS patients examined. Interestingly a larger proportion of patients with rheumatoid arthritis expressed such activity (over 20%).

5.1.3 Can APL cause functional changes in the fibrinolytic system?

The mere presence of antibody activity directed against proteins in the fibrinolytic pathway in patients with APS does not necessarily imply a role in the pathogenesis of the condition for such proteins. It is much more convincing to demonstrate a change in functionality of the target proteins. Recently evidence has come to light that the sera of patients with APS may possess such properties.

The Fab portion of IgG fraction of patients with APS has been shown to impair the rate of dissolution of fibrin clots by plasmin under both static and flow conditions (Kolev *et al*, 2002). The results of Kolev *et al* also suggested that aPL have a direct effect on the interaction of plasmin with fibrin. Interestingly the IgG fraction of normal plasma was found to prolong clot lysis time in the system used here but to a lesser extent to that seen for aPL IgG fractions. Evidence from patients with myeloma suggests that this effect may relate to change in the size of fibrin fibres formed in the presence of IgG (Carr, Jr. *et al*, 1996).

Some workers have examined the effect of monoclonal antibodies possessing aPL activity on fibrinolytic function *in vitro*. β2GPI has been shown to protect tPA from the inhibitory action of PAI I, this effect has been shown to be reduced in the presence of monoclonal IgM antibodies derived from a patient

with APS (Ieko *et al*, 1999) (Ieko *et al*, 2000). It is interesting to compare these results, which show aPL acting indirectly on tPA to those discussed earlier presenting evidence of a direct tPA interaction. One must remember however that the monoclonal antibodies used in this system are unlikely to represent the full repertoire of activity seen in the sera of an APL patient with polyclonal antibodies. As well as functional changes in extrinsic fibrinolysis, monoclonal antibodies with APL activity have also been demonstrated to impair the activity of the intrinsic fibrinolytic system (Takeuchi *et al*, 2002).

Anti-plasmin antibody activity has also been demonstrated in the sera of APS patients. Yang *et al* describe 28% of their APS patient cohort possessing such activity. This group went on to examine the function of anti-plasmin activity using monoclonal aPL Ig with anti-plasmin activity. They were able to demonstrate a reduction in fibrin plate lysis related to one of the monoclonal antibodies studied (Yang *et al*, 2004).

More recent work has focused on the interaction of aPL with components of the pericellular fibrinolytic system. Cesarman-Maus *et al* have recently described the presence of antibodies with anti-Annexin II activity in the sera of patients with APS. These were significantly increased in patients with APS compared to individuals with SLE or normal controls. Moreover, affinity purified antibodies with anti-Annexin II activity were shown to posses potentially pathogenic activities. These included inducing an upregulation of TF in HUVEC, reducing tPA dependent plasminogen activation in the presence of annexin II and reducing cell surface associated plasminogen activation at the HUVEC surface (Cesarman-Maus *et al*, 2006). Some of these effects were independent of anti-β2GPI activity and did not require β2GPI as a cofactor. Patterson *et al* have

recently demonstrated reduced pericellular fibrinolytic activity in endothelial cells pre-incubated with aPL positive sera using a cellular based fibrin clot lysis assay (Patterson *et al*, 2006). Four of fourteen samples tested expressed this activity. The authors of this study suggested increased PAI-1 activity in aPL exposed cells as the reason for this effect.

5.1.4 Aims of this section

1) To examine the effect of affinity purified IgG fractions from patients with APS on the binding of plasminogen to endothelial cell surfaces and determine whether any affect observed could be modulated by cell activation.

2) To determine whether IgG from patients with APS could impair fibrin lysis in a plasma clot lysis assay activated by uPA (previous studies have used tPA as an activator).

5.2 A study into the interaction of aPL and plasminogen at the endothelial cell surface

5.2.1 Provisional experiments on the binding of plasminogen to HUVEC in the presence of aPL using flow cytometry

To test for an effect on plasminogen binding to HUVEC I first looked at the effect of aPL in an experimental system using flow cytometry to quantify binding of FITC labelled plasminogen (Biomac®) (FITC-Pg). HUVEC were cultured as described in the materials and methods section in $25cm^2$ flasks to confluence. For all experiments, the cells were at passage 5 or less. The cells were removed from the flask using Accutase® and after resuspension in culture media and centrifugation (200g for 10 minutes), were resuspended in 150μl of HBSS. 20μl of cell suspension was then incubated for 45 minutes with 45μl Dulbeco's PBS, 100μl of IgG fraction from a patient with APS or pooled normal plasma (final concentration 0.59mg/ml), 5mcl of FITC-Pg in water (final concentration 29μg/ml). For each run of experiments, a duplicate arm was included in which EACA was added to the PBS (final concentration 26mM). The contents of each tube were then suspended in 1 ml of HBSS and analysed on a coulter-epics flow cytometer using the FL1 channel. The details of the gating strategy used and an example of a typical HUVEC scatter plot are shown in the materials and methods section. For each experiment, at least 10,000 events within the endothelial cell gate were analysed. The mean fluorescence intensity of all events in the endothelial cells gate for each arm was recorded. Each experimental arm was repeated in triplicate. Results for patients 1, 2 and 3 are shown in figure 49, example histograms for patient 3 are shown in figure 48.

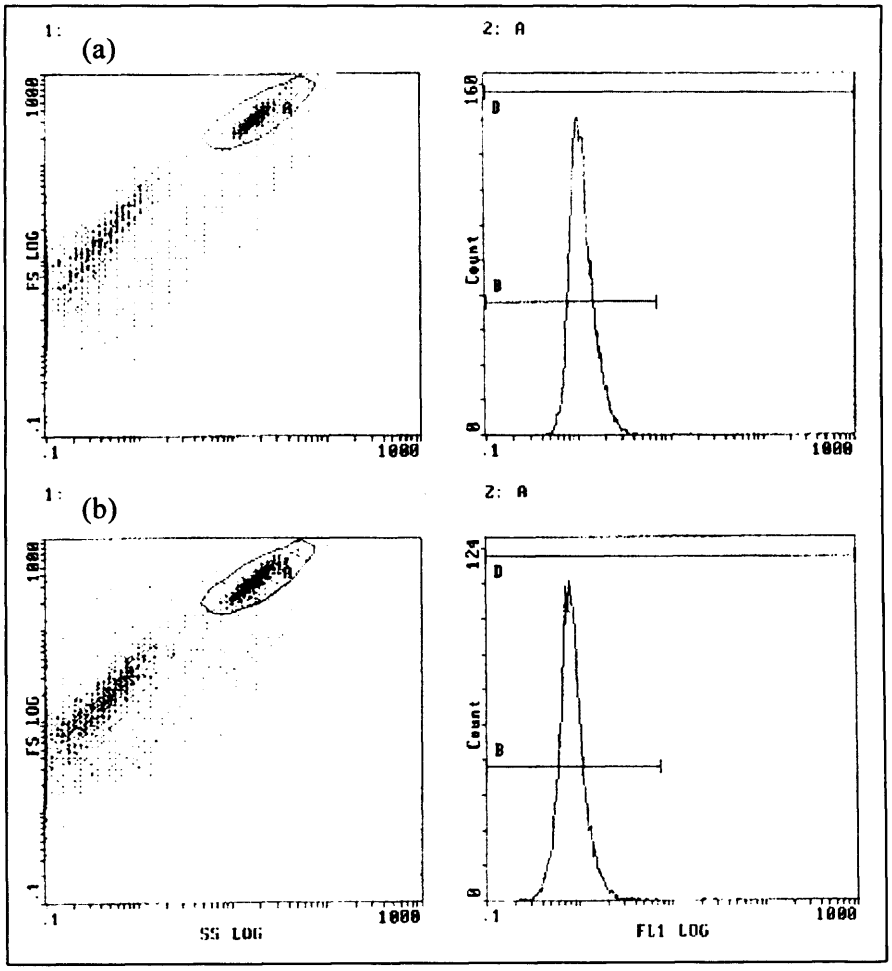


Figure 48 Example scatter plot and histograms for HUVEC incubated with FITC-plasminogen

(a) HUVEC incubated with IgG from PNP (b) HUVEC incubated with IgG from patient 3. Endothelial cells were gated in region A as discussed in the general methods section. The mean fluorescence intensity within region D was recorded. The mean fluorescence intensity in (a) was 1.34 and in (b) 0.98.

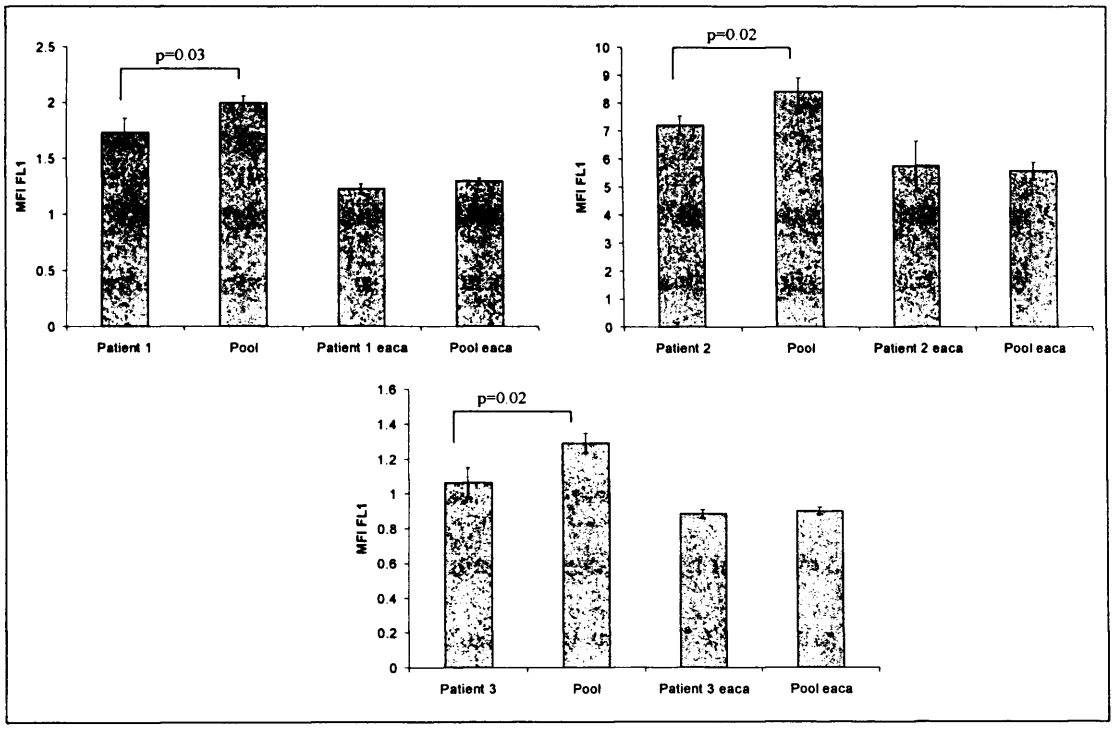


Figure 49 Binding of FITC-Pg in the presence of IgG fractions from patients with aPL and pooled normal plasma.

Results of the mean and standard deviation for triplicate experiments are shown. In each case a significant difference was seen in an independent sample t-test between aPL and non-aPL exposed cells. No significant difference was observed in binding in the arms with EACA present. MFI = mean fluorescence intensity.

In each of the experiments illustrated above a small but statistically significant reduction in binding of FITC-Pg was observed in the presence of aPL. For these initial experiments, I chose patients in our cohort with some of the highest aPL levels. I wanted to extend the analysis of this reduction in binding further. One problem however with analysis of endothelial cells in this system is that because the cells are in "liquid phase" during analysis, the binding of FITC-Pg to both what would have been the cells basolateral and luminal surfaces is analysed. To circumvent this problem, I set up experiments to assay the binding of plasminogen to the HUVEC "luminal" surface using a fluorescent plate

reader. The development of these assays and the results are presented in the sections which follow.

5.2.2 Provisional experiments on the binding of plasminogen to HUVEC using a fluorescent plate reader system.

The manufacturer and technical details of the fluorescent plate reader used in these experiments is given in the materials and methods section. HUVEC were cultured on Povair 215006 96 well plates. The 96 well plates were coated in 1% gelatin prior to cell culture. The contents of an $80cm^2$ culture flask at passage 5 or less with confluent HUVEC was removed with Accutase® and after resuspension in media and centrifugation, resuspended in 20mls of culture media. 200µl of this cell suspension was added to wells of the gelatin coated 96 well plate, for experiments involving a non-cell control arm 200µl of culture media alone was added. (Note wells without cells were also gelatin coated). HUVEC were grown to confluence in each well before being used in experiments.

Because of previous problems with cell loss during washing steps, for all of the following experiments, the cells were fixed in 1% paraformaldehyde (PFA) for 20 minutes before binding assays were carried out. Cells were washed three times in Dulbeco's PBS after fixing.

For all experiments assaying binding of FITC-Pg, the plate reader was set to an excitation wavelength of 485nm and an emission wavelength of 520nm. The plate reader gain was set against the emission of the brightest well in each experiment unless otherwise stated.

An initial experiment was performed to examine the binding of FITC-Pg over a range of concentrations. A previously prepared Povair 215006 96 well plate with confluent HUVEC and wells with media only was used. Cells were

fixed with 1% PFA prior to binding studies. The wells without cells were also exposed to PFA. In triplicate, cell containing and non-cell containing wells were incubated for 30 minutes with 50µl of FITC-Pg in Dulbeco's PBS over a range of 5 doubling dilutions (final initial concentration 16.7µg/ml) with or without EACA (final concentration 100mM) and 150µl of Dulbeco's PBS. After incubation at room temperature for 30 minutes (with the plate wrapped in foil) the wells were washed 3 times with Dulbeco's PBS with final residual volume of 100µl PBS added at the end. The binding of FITC-Pg was then assessed using a fluorescent plate reader (excitation wavelength of 485nm emission wavelength 520nm). The results of this experiment are shown in figure 51.

As well as examining the binding of plasminogen labelled with FITC to the HUVEC monolayer, I also set up a chromogenic assay to demonstrate the difference in binding between lys-plasminogen and glu-plasminogen at the fixed HUVEC surface. HUVEC were grown to confluence on a Costar 3595 96 well cell culture plate as described in the general methods section. Cells were then fixed in 1%PFA as above and washed. Fixed cell monolayers were then exposed in triplicate for 30 minutes to 50µl/well of lys-plasminogen or glu-plasminogen (Enzyme Research) (final concentration 16.5 µg/ml in PBS) and 50µl/well of PNP IgG at 1mg/ml (final concentration 0.5 mg/ml). Cells were then washed three times in PBS and then incubated at 37°C with 100µl/well plasmin substrate (H-D-Val-Leu-Lys-pNA Channel Diagnostics Ltd) final concentration 0.54 mg/ml and 50µl/well tPA (Quadratech) final concentration 17ng/ml. The OD at 405nm was read every 10 minutes. Results of a representative experiment are illustrated in figure 50 and show reduced activity of glu-plasminogen compared to lys-plasminogen at the cell surface.

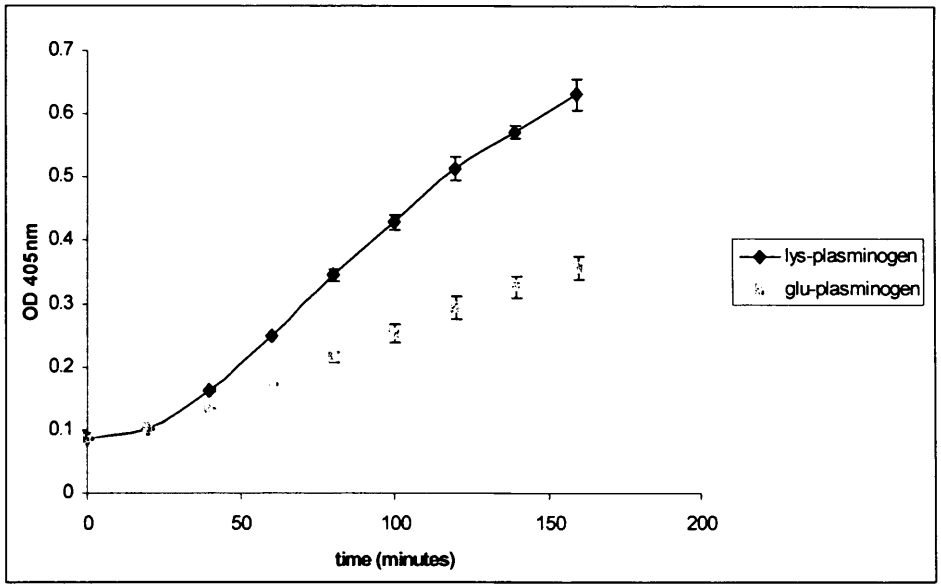


Figure 50 Comparison of activity of glu-plasminogen and lys-plasminogen at fixed HUVEC monolayers

Results of triplicate wells and standard deviations are shown. Details of the chromogenic assay used are given in the main text.

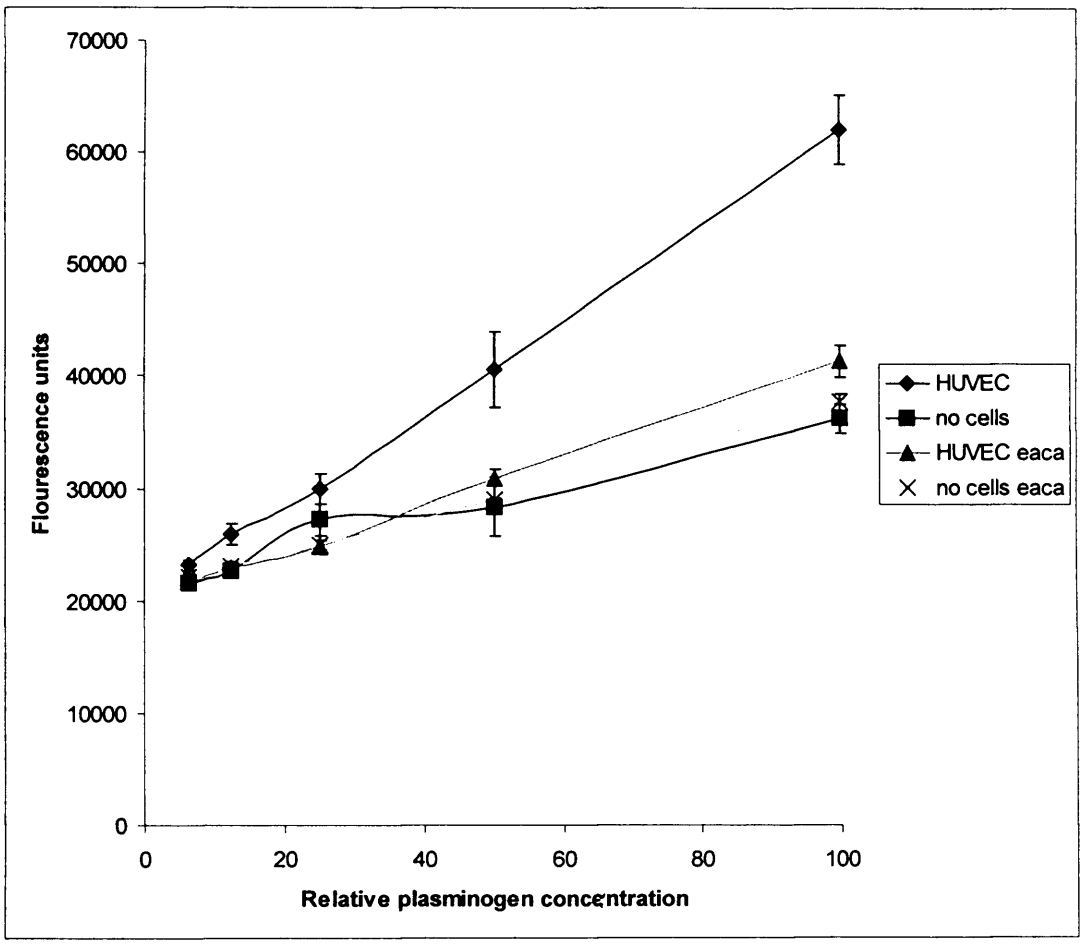


Figure 51 Binding of FITC-Plasminogen to HUVEC coated and non-cell coated wells in the presence and absence of EACA.

A relative plasminogen concentration of 100 corresponds to a final concentration of 16.7μg/ml. Results of the mean and standard deviation are shown for triplicate wells.

The previous data illustrated a reduction of binding of plasminogen to the HUVEC to very near that of no cells in the presence of EACA. To test whether the presence of immunoglobulin could interfere with the binding of plasminogen in this system I ran a second binding experiment in the presence and absence of IgG fraction from pooled normal plasma. The reaction conditions were as above with the exception that the plasminogen binding step was performed in the presence of IgG from pooled normal plasma (final concentration 0.5mg/ml). The results (shown in figure 52) showed no significant difference in binding of plasminogen in the presence and absence of IgG.

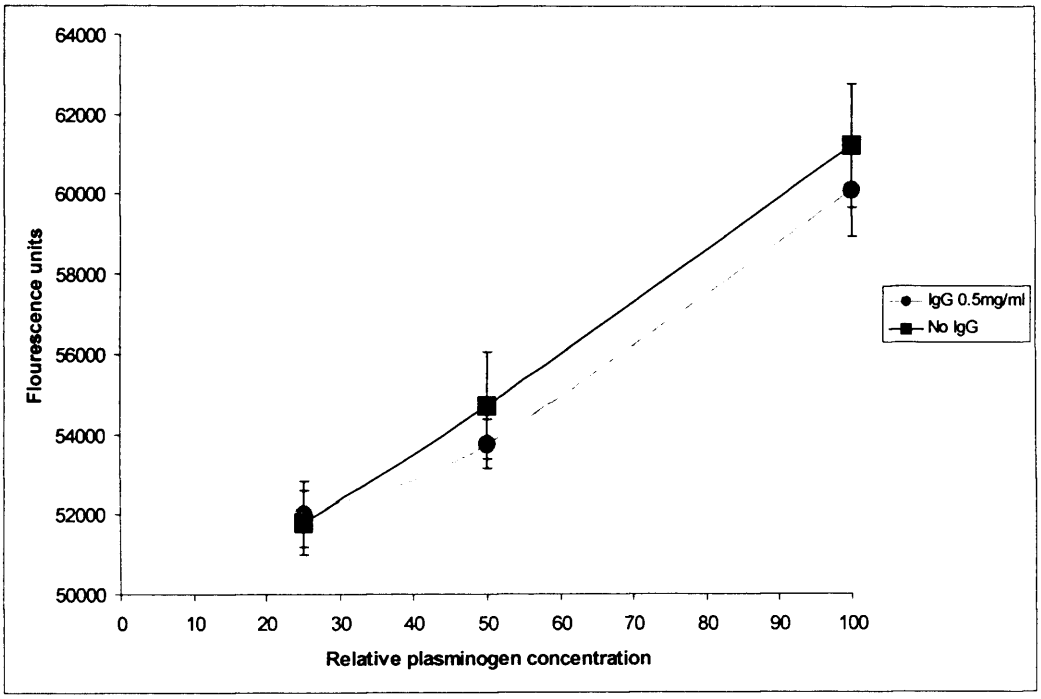


Figure 52 Binding of FITC-Pg to HUVEC in the presence and absence of IgG

A relative plasminogen concentration of 100=16.7μg/ml. The mean and standard deviation of quadruplicate wells are shown.

5.2.3 An examination of the effect of the presence of aPL on the binding of Plasminogen to the endothelial cells surface.

To examine whether aPL could affect the binding of plasminogen to the endothelial cell surface in a monolayer the following assay was set up for nine different patient IgG fractions. HUVEC at confluence on a gelatin coated Povair 215006 96 well plate were fixed with 1%PFA. After washing wells, groups of six replicates were incubated for 30 minutes with FITC-Pg (final concentration 16.7μg/ml); IgG fraction in Dulbeco's PBS (either patient with aPL derived or from pooled normal plasma, final concentration 0.5mg/ml); with or without EACA (final concentration 100mM). After incubation for 30 minutes at room temperature with the plate wrapped in foil, wells were washed three times in Dulbeco's PBS with a final residual volume of 100μl added at the end. Binding of FITC-Pg was then measured on a fluorescent plate reader (excitation wavelength of 485nm emission wavelength 520nm). The results for the nine patient IgG fractions examined are shown in table 20.

The anti-β2GPI activity of the patient IgG fractions was checked using the modified anti-β2GPI assay described in the last chapter. Results are illustrated in figure 53.

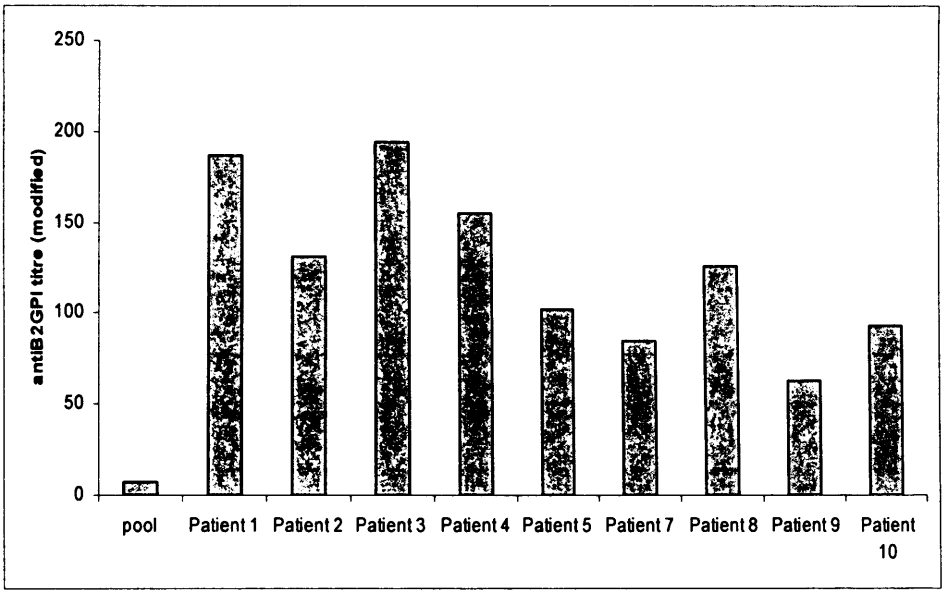


Figure 53 Modified anti-β2GPI assay results for the patent IgG fractions used in this section.

	Pool	Pool eaca	Patient 1	Patient 1 eaca
mean	58892.83	52242.33	58175.83	52215.17
sd	1347.59	1196.45	2029.02	958.55
%cv	2.29	2.29	3.49	1.84

	Pool	Pool eaca	Patient 5	Patient 5 eaca
mean	56973.17	50476.50	55253.17	50421.83
sd	1936.49	1855.34	1614.35	1659.22
%cv	3.40	3.68	2.92	3.29

	Pool	Pool eaca	Patient 2	Patient 2 eaca
mean	58117.67	48165.83	56104.00	48053.83
sd	1456.33	884.97	1756.79	1464.21
%cv	2.51	1.84	3.13	3.05

	Pool	Pool eaca	Patient 7**	Patient 7 eaca
mean	60035.83	47009.67	55533.00	47271.67
sd	2221.10	1099.58	2465.05	1275.50
%cv	3.70	2.34	4.44	2.70

	Pool	Pool eaca	Patient 3*	Patient 3 eaca
mean	55145.17	44736.17	51263.33	45286.83
sd	1695.07	1031.31	1142.19	1460.30
%cv	3.07	2.31	2.23	3.22

	Pool	Pool eaca	Patient 8	Patient 8 eaca
mean	59809.33	52717.00	59358.83	54093.67
sd	1640.32	1314.46	2428.76	1714.65
%cv	2.74	2.49	4.09	3.17

	Pool	Pool eaca	Patient 4	Patient 4 eaca
mean	55318.17	50960.67	54341.50	51460.17
sd	2140.77	1601.66	1589.06	805.15
%cv	3.87	3.14	2.92	1.56

	Pool	Pool eaca	Patient 9	Patient 9 eaca
mean	58865.67	54853.00	59388.50	55188.83
sd	711.33	1887.59	2290.55	1137.86
%cv	1.21	3.44	3.86	2.06

	Pool	Pool eaca	Patient 10	Patient 10 eaca
mean	60106.67	54641.67	59659.33	55717.00
sd	1785.53	1383.29	2027.75	2331.43
%cv	2.97	2.53	3.40	4.18

Table 20 Fluorescence unit readings for FITC-Pg binding to HUVEC in the presence and absence of aPL

In none of the cases was a significant difference observed in binding between the aPL and pooled plasma IgG fractions in the presence of EACA. For patients 3 and 7 (*/**), a significant reduction in binding was noted in the non-EACA exposed arms in the presence of aPL containing IgG fractions. (p= 0.0009 and 0.008 respectively). The results show the mean, standard deviation and % coefficient of variation for six HUVEC coated wells in each experimental arm.

For two of the patient IgG fractions tested (patients 3 and 7), a small but statistically significant reduction in plasminogen binding was noted in the presence of IgG containing aPL activity. Interestingly patients 1 and 2 who had previously shown a reduction in binding in the experiments using a flow cytometer failed to demonstrate any difference in this system. This may relate to the fact that only the "luminal" side of the cells is examined in this assay or could relate to the utilisation of fixing with PFA changing the binding characteristics of the cells for aPL and /or plasminogen. The results for the patient IgG samples showing a significant effect are shown in figure 54.

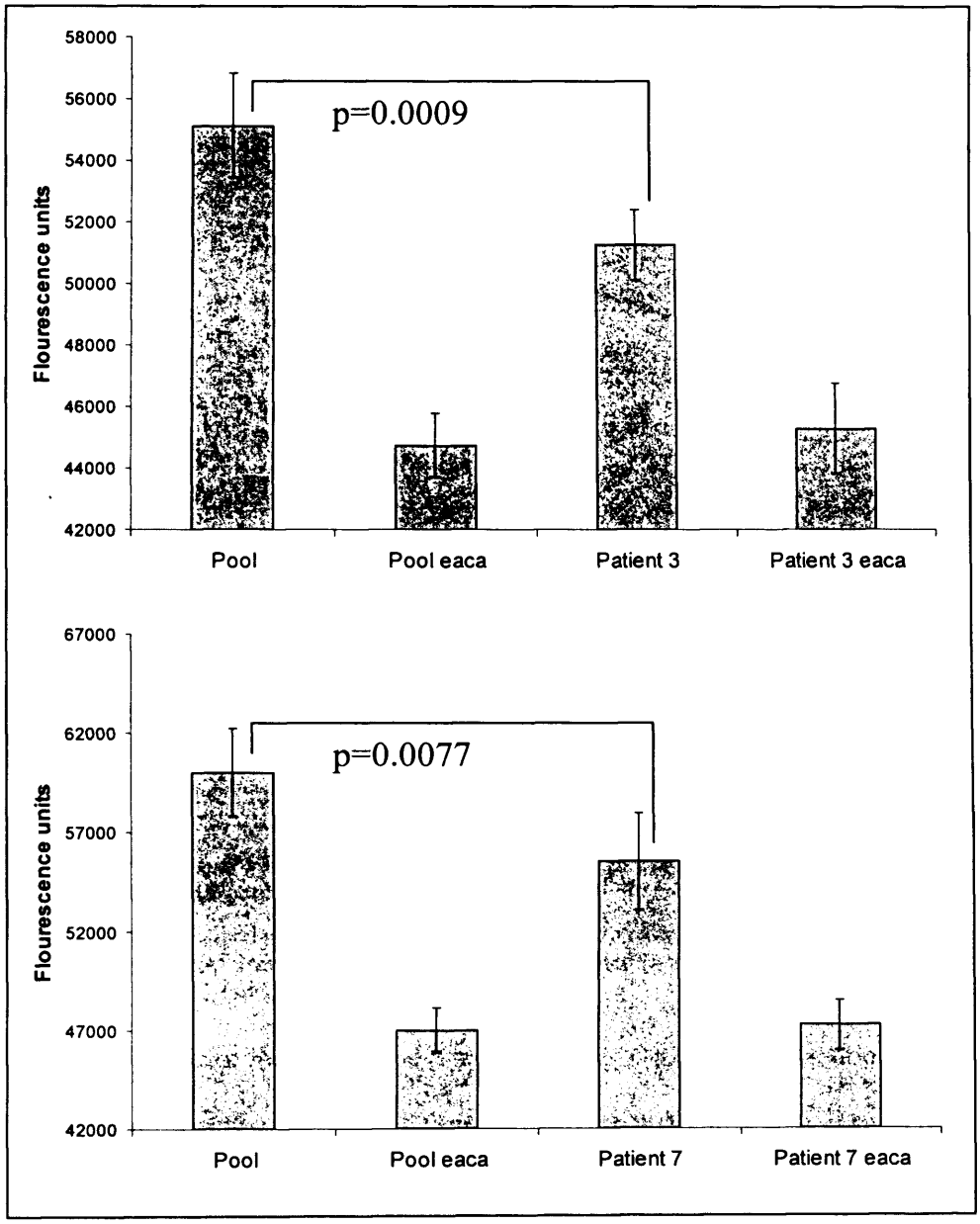


Figure 54 Binding of FITC-Pg to HUVEC in the presence of IgG fractions from pooled normal plasma and patients 3 and 7.

Results show the mean and standard deviation for six replicate wells in each experimental arm. The p value is derived from an independent samples 2 tailed t-test. No significant difference was observed between aPL and pool plasma IgG arms exposed to EACA.

To test whether this effect of reduction in plasminogen binding in the presence of aPL exhibited change in the presence of different concentrations of aPL, I set up a further experiment with the same conditions as above with different concentrations of IgG fractions from pooled normal plasma and patient 7. The (final) antibody concentrations in the assay were 1mg/ml, 0.5mg/ml and

0.25mg/ml. The results of this dose response experiments are shown in figure 55 below.

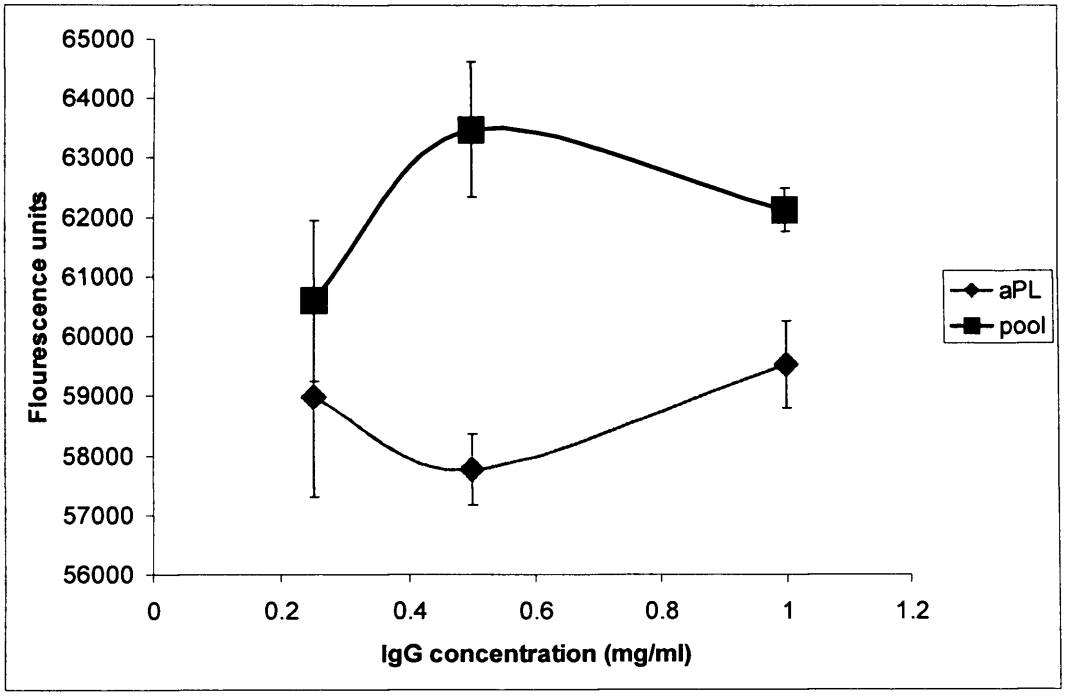


Figure 55 Binding of FITC-Pg to HUVEC monolayer in the presence of IgG fraction from patient 7 (aPL) and pooled normal plasma.

Mean results and standard deviations are shown for triplicate HUVEC coated wells in each experimental arm. At 1mg/ml and 0.5 mg/ml, the difference in binding was significant (p=0.005 for both arms on a 2-tailedindependent samples t test).

The results above showed a trend towards maximum reduction in binding at 0.5mg/ml. For the results at 0.25mg/ml IgG no difference was seen in binding between however much of this is due to a reduction in binding in the pooled plasma IgG fraction arm rather than an increase in binging in the aPL fraction arm. Of note however the variation was much wider at this concentration of IgG than for the two higher concentrations.

5.2.4 Can the binding of plasminogen to the endothelial cell surface in the presence of aPL be modulated by activating the cells?

Given the observation that changes in the endothelial binding properties of aPL have been observed depending on the activation state of the cells (Chen *et al*, 2004) I thought it worthwhile to test whether activation of HUVEC could lead to a change in the interaction of plasminogen and aPL IgG at the HUVEC surface.

To test whether this was the case, I set up a system to activate HUVEC using TNFα (PePro Tech Inc). A provisional experiment was performed to look for changes in endothelial cell activation markers following 20 hours stimulation with 100units/ml TNFα. The parameters examined were surface expression of ICAM-1, VCAM and thrombomodulin by flow cytometry. HUVEC at confluence in a $25cm^2$ flask under culture conditions as described in the general methods section were exposed for 20 hours to TNFα (100 units/ml) in culture media. The cells were then mobilised from the culture flask with Accutase®, resuspended in media and after centrifugation the cell pellet resuspended in 150μl HBSS. 20μl of cell suspension was added to each tube. Cells were then incubated with test and isotype control monoclonal antibodies for 15 minutes before addition of 500μl HBSS (with the exception of the thrombomodulin arm where a secondary antibody was used to detect primary antibody binding). Test and control antibody concentrations were equal and volumes added were in accordance with the manufacturer's instructions. The mean fluorescence intensity (MFI) of the HUVEC was then recorded for each test and control arm for 10,000 events (gating strategy as described in the general methods section). The FL1 channel was used for FITC conjugated antibody and the FL2 channel

for PE conjugates. Results were expressed as MFI test antibody – MFI isotype control antibody. The details of the monoclonal antibodies used are shown in table 21. The change in MFI between TNFα cells is shown in figure 56.

ICAM-1 Test antibody: Mouse IgG1 PE (Immunotech) Control antibody: Mouse IgG1 PE (Immunotech) VCAM Test antibody: Mouse IgG1a FITC (Research Diagnostica) Control antibody :Mouse IgG1a FITC (Immunotech) Thrombomodulin Primary antibodies: Test antibody: Mouse IgG1 (Research Diagnostica) Control antibody: Mouse IgG1 (Dako) Secondary antibody: Goat F(ab')2 anti mouse FITC (Dako)

Table 21 Monoclonal antibodies used in this section.

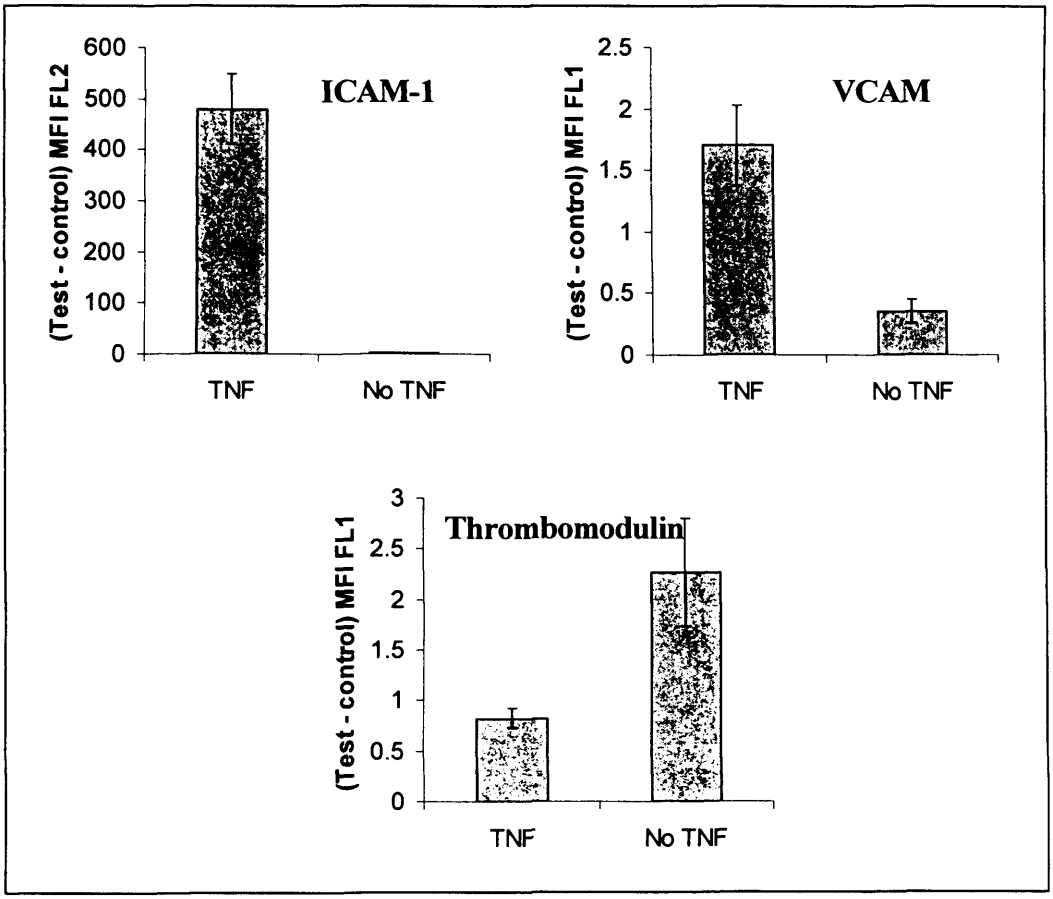


Figure 56 Expression of ICAM-1, VCAM and thrombomodulin following stimulation of HUVEC with TNFα for 20 hours.

A significant difference in expression was noted for ICAM-1, VCAM and thrombomodulin surface expression (p=0.0003, 0.002 and 0.01 respectively on an independent sample 2 –tailed t-test). The results shown represent the mean and standard deviations for three experiments in each arm.

Having established that the HUVEC activated in this system with 100units/ml TNFα, I transferred the activation to the 96 well fluorescence culture plate system. HUVEC were cultured on Povair 215006 96 well plates as previously described. Once confluent, some of the cells were exposed to 20hours of stimulation with culture media containing 100units/ml TNFα. Initially I checked whether this stimulation resulted in any change in the binding of FITC-Pg to the cell surface. Cells were fixed in 1% PFA prior to binding experiments with FITC-Pg and read on a fluorescent plate reader as previously described. The final concentration of FITC-Pg was 16.7μg/ml, no IgG fraction was added to any

arm. Final concentration of EACA was 100mM. The results of this experiments are shown in figure 57.

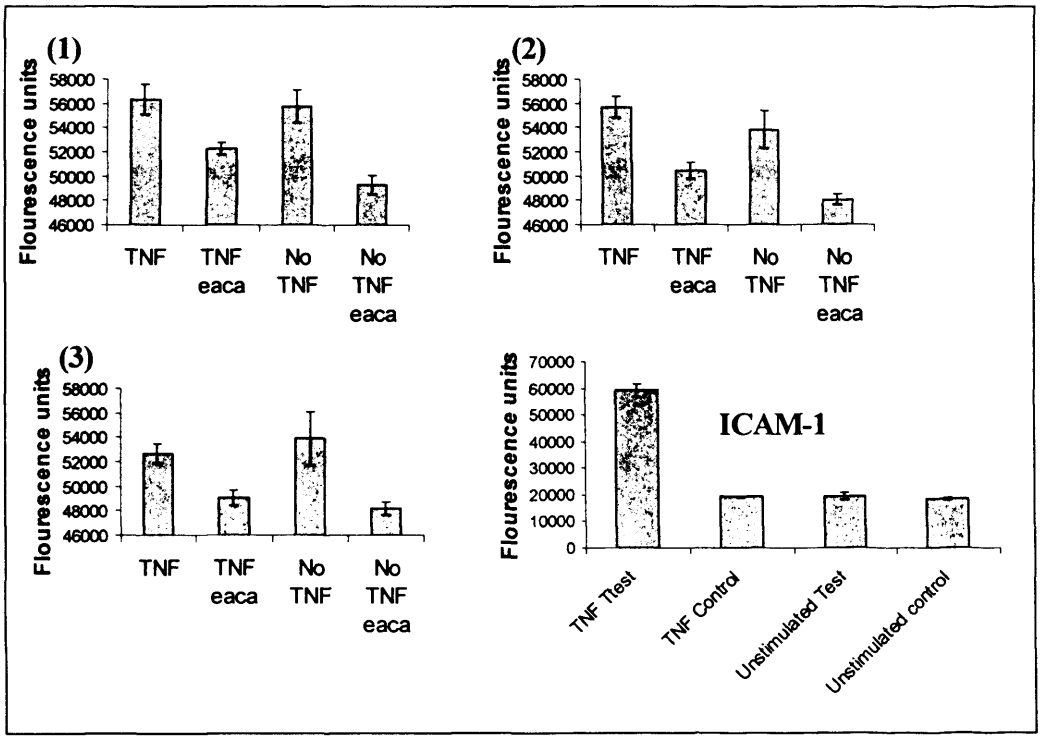


Figure 57 Results for FITC-Pg biding to TNFα activated and non-activated HUVEC.

Results are shown for three experimental runs. Mean and standard deviations for quadruplicate wells are shown. No significant difference was observed for total FITC-Pg binding in TNFα activated and non-activated arms. In experiments (1) and (2) a significant difference was noted in binding in the EACA exposed arms between the TNFα and non-TNFα exposed arms (p=0.0005 and 0.001 respectively). In experiment (3) this difference did not reach significance (p=0.08). Also illustrated is the increase in ICAM-1 expression seen in the TNFα stimulated HUVEC in the 96 well plate system (test and control antibodies were as per the flow cytometry experiments described above; antibody was diluted in 1% albumin Dulbeco's PBS; incubation for 30 minutes; cells washed three times in PBS prior to reading (plate reader settings for PE: excitation 544nm; emission 590nm); results of mean and standard deviation for quadruplicate wells are shown.

The results above showed no difference in total binding of FITC-Pg to TNFα exposed HUVEC. They did however show a trend towards, and increase in, non-specific binding of plasminogen illustrated by the increased binding in the EACA exposed arms in two out of three of the experimental runs. This could imply an increase in non-lysine dependent binding with a decrease in lysine-specific binding in TNFα activated cells given that there is no apparent change in total binding seen.

I next tested the binding of plasminogen to TNFα activated cells in the presence of IgG fraction from a patient previously shown to reduce plasminogen binding in previous experiments (patient 7). Results are shown below illustrating that the effect of a reduction in binding in the presence of aPL is still seen after TNFα stimulation (figure 58). The second figure (figure 59) illustrates no difference in total binding in the presence of aPL pre and post TNFα, however the small increase in binding in the presence of EACA is still noted in this experiment.

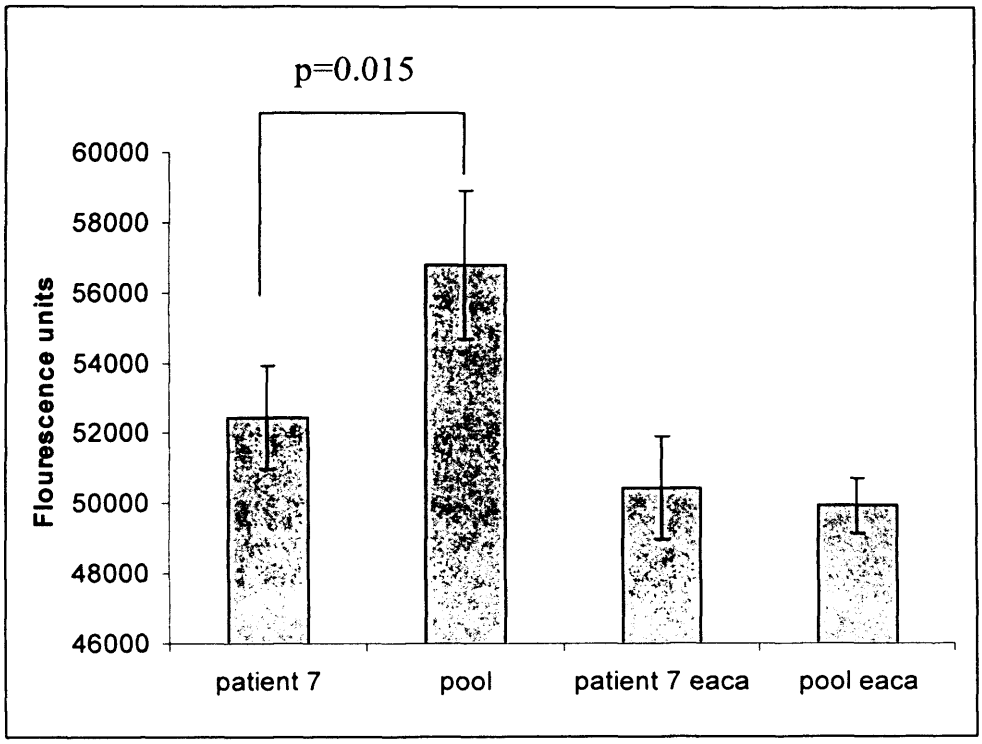


Figure 58 Binding of FITC-Pg to TNFα stimulated HUVEC in the presence IgG fraction from patient 7 and pooled normal plasma.

As with the non-TNFα exposed cells, a significant difference is noted between the APA exposed and non-aPL exposed cells. No significant difference was seen between the two EACA exposed arms. Mean and standard deviations are shown for quadruplicate HUVEC coated wells in each arm. P values for a 2-tailed independent samples t-test are shown.

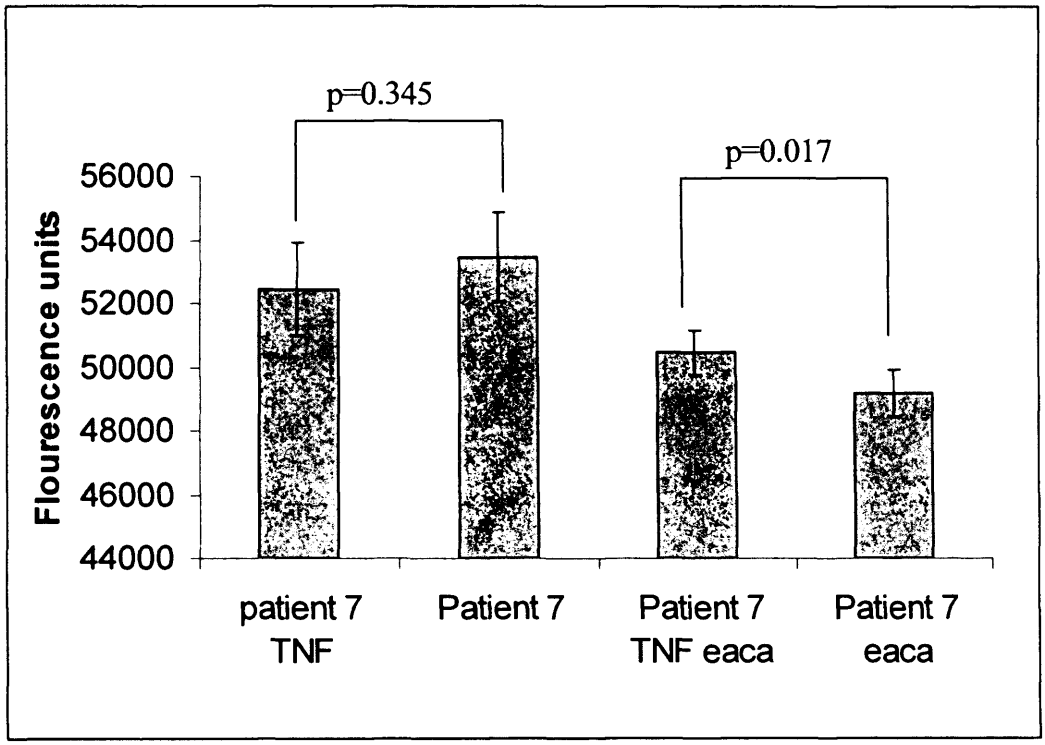


Figure 59 Comparison of FITC-Pg binding in the presence of aPL containing IgG fraction pre and post stimulation of HUVEC with TNFα

Results of the mean and standard deviations for quadruplicate HUVEC coated wells in each arm are shown. P values for an independent samples t-test are shown.

With these results in mind, I then tested the binding of FITC-Pg to TNFα stimulated HUVEC in the presence of the IgG fractions which had previously not shown any difference in binding compared to IgG fraction from pooled normal plasma. The fixing and binding assay was as previously described, results are shown in table 22. None of the patient samples showed any significant reduction in binding compared to pooled normal plasma IgG fraction.

	Pool	Pool eaca	Patient 1	Patient 1 eaca
mean	56594.00	50967.00	57131.17	49953.25
sd	1811.58	783.67	1299.13	872.25
%cv	3.20	1.54	2.27	1.75

	Pool	Pool eaca	Patient 5	Patient 5 eaca
mean	53060.17	50429.00	52175.00	51121.00
sd	1668.71	1041.49	1466.69	820.81
%cv	3.14	2.07	2.81	1.61

	Pool	Pool eaca	Patient 2	Patient 2 eaca
mean	60294.17	52918.75	58477.17	56083.00
sd	1839.71	9363.15	1678.69	1049.79
%cv	3.05	17.69	2.87	1.87

	Pool	Pool eaca	Patient 8	Patient 8 eaca
mean	54533.67	51768.00	56172.67	52817.50
sd	839.18	1737.33	960.84	1301.67
%cv	1.54	3.36	1.71	2.46

	Pool	Pool eaca	Patient 4	Patient 4 eaca
mean	56635.00	54893.25	57280.67	54325.75
sd	728.49	1601.93	1059.87	369.45
%cv	1.29	2.92	1.85	0.68

	Pool	Pool eaca	Patient 9	Patient 9 eaca
mean	58642.67	55759.50	59291.67	56023.25
sd	1620.03	976.77	1064.71	267.39
%cv	2.76	1.75	1.80	0.48

	Pool	Pool eaca	Patient 10	Patient 10 eaca
mean	56722.33	53102.00	55491.33	54358.00
sd	1258.82	1311.96	1324.10	1473.22
%cv	2.22	2.47	2.39	2.71

Table 22 Results for FITC-Pg binding to activated HUVEC in the presence of IgG fraction from Pooled normal plasma and patients with APS.

No significant difference in binding of plasminogen between PNP and patient exposed IgG arms were seen for any of the samples examined.

5.2.5 Is the reduction in plasminogen binding seen related to the level of binding of APA to the HUVEC surface?

To examine whether the reduction in binding of FITC-Pg to the HUVEC surface was related to the degree of binding of the antibodies to the cells, I set up an assay to detect the level of binding of the antibodies themselves to HUVEC.

HUVEC were plated onto gelatin coated Povair 215006 96 well plates as previously described and experiments performed once the cells were confluent. After fixing with 1%PFA, the cells were incubated in triplicate with 1mg/ml (50μl/well) IgG fractions from Pooled normal plasma and patients with APS in quadruplicate. After 30 minutes, the cells were washed in PBS and incubated for 30 minutes with mouse anti-human IgG (Immunotech 0279) at 4μg/ml (50μl/well). The cells were then washed three times in PBS and incubated for 30 minutes with goat anti-mouse F(ab)' FITC conjugated (Dako F0479, diluted 12.5x in PBS) 50μl/well. After washing, the fluorescence intensity of each well was measured on a fluorescent plate reader (excitation wavelength of 485nm and

emission wavelength of 520nm). The results of this experiment are shown in figure 60.

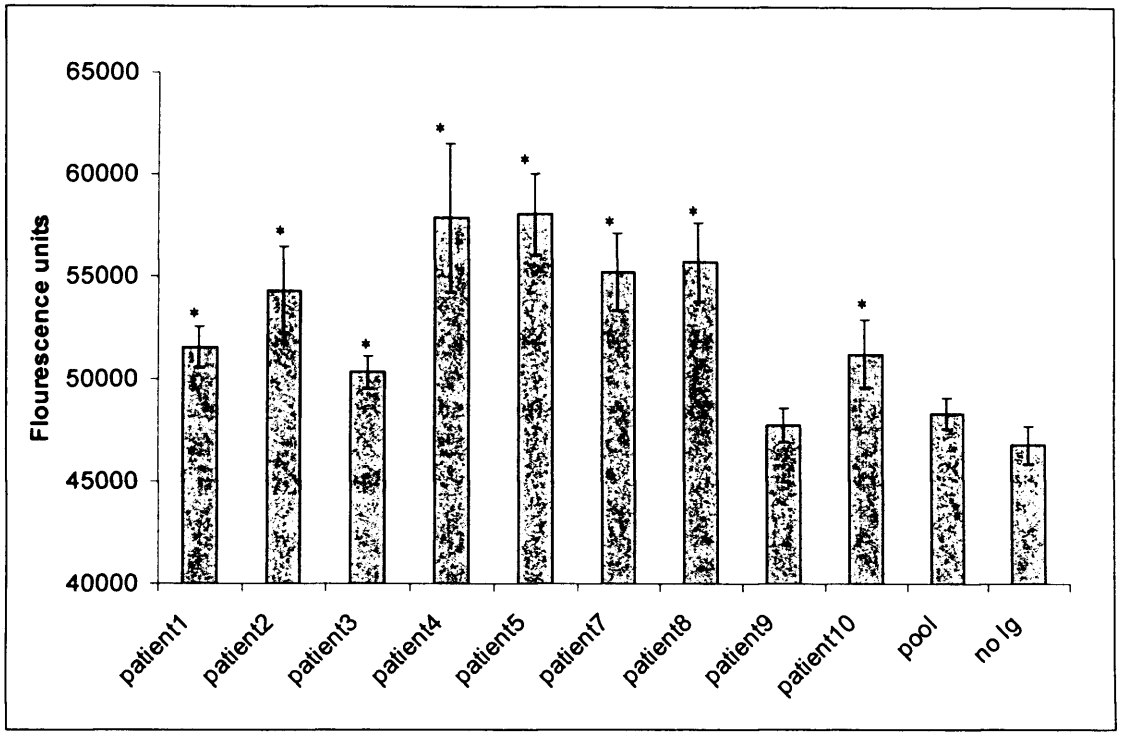


Figure 60 IgG binding to HUVEC for patient IgG fractions with aPL activity and pooled normal plasma.

Results are shown for the mean and standard deviation of quadruplicate HUVEC coated wells. * indicates a p-value of <0.05 for a comparison between the pooled normal plasma IgG and patient IgG in a 2-tailed independent samples t-test.

The results of this experiment illustrate that all of the aPL containing IgG fractions examined except for that from patient 9 exhibit increased binding to the surface of HUVEC compared to IgG from pooled normal plasma. Notably however this effect does not seem to correlate with the IgG fraction causing impairment of binding of plasminogen to the cell surface.

5.3 An investigation into the effect of aPL on the lysis of clotted plasma

To date, only a few studies have examined the effect of aPL on the dissolution of clots in a microtitre plate lysis system (Kolev *et al*, 2002) (Yang *et al*, 2004). The previous work of Kolev *et* al had used tPA to stimulate clot lysis, I decided to see whether a uPA stimulated system could produce a similar effect. Given that earlier work in this thesis had shown a small but nevertheless present reduction in aspects of fibrinolytic activity in the presence of aPL from some of the patients studied, I wanted to see whether any effect could be discerned in a global assay of fibrinolytic activity.

5.3.1 Preliminary plate lysis experiments

To examine the effect of aPL on the lysis of clots, I set up an assay to monitor the reduction in optical density of clotted plasma as fibrinolysis proceeds. 20μl of high molecular weight urokinase (uPA) (Calbiochem) (1.5μg/ml in PBS), 20μl of bovine thrombin (Diagnostic reagents Ltd.) (16U/ml in 0.9% saline) and 50μl of IgG fraction from pooled normal plasma or a patient with APS (1mg/ml in PBS) were added to the well of a microtitre plate (96F microplate FX9200 Alpha laboratories) and mixed. 50μl of coagulation reference plasma (Technoclone®, double diluted with water) was then added to the well and the reagents mixed on a plate mixer. (Final concentrations: thrombin 2.3U/ml; uPA 0.2μg/ml; IgG 0.36mg/ml). Reagents were kept in ice prior to adding to the plate. The plate was then incubated at 37°C and the optical density of each well measured at regular time intervals at 405nm. In each experiment, samples were run in triplicate or quadruplicate as stated.

In these experimental runs which were performed initially using IgG fraction from patient 3 (previously found to be active in other assays of fibrinolytic activity), I found that while the intra-assay variation in clot lysis times was small, the inter- assay variation was large. This may have been due to differences in timing between the additions of the reagents to the microtitre plate before incubation at 37°C. This meant that I could not easily compare 50% clot lysis times between experiments. Instead, I opted to look at the reduction in OD at 405nm at the peak and mid point of the lysis curve and compare that for the aPL IgG fraction and pooled normal plasma IgG fraction exposed arms. An example of two of these experiments shown in figure 61.

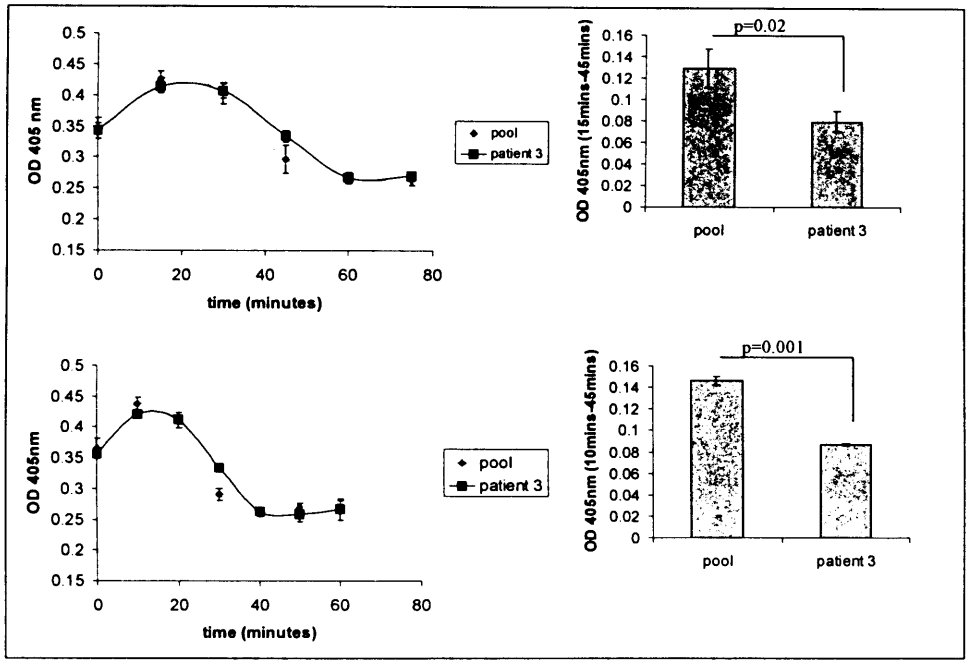


Figure 61 Plate lysis curve and difference in OD405nm at peak and midpoint of curves for IgG fraction from patient 3 and Pooled normal plasma.

The mean and standard deviation for triplicate wells are shown in each graph. The p value is for a 2-tailed independent samples t-test. A reduction in clot lysis is seen in both experimental arms exposed to IgG fraction from patient 3.

To detect whether this effect was abrogated by a reduction in IgG concentration, I re-ran this provisional experiment but this time included an arm

with IgG fractions at 50% of the concentration used in the preceding experiments. This showed a reduction of the effect at lower IgG concentrations.

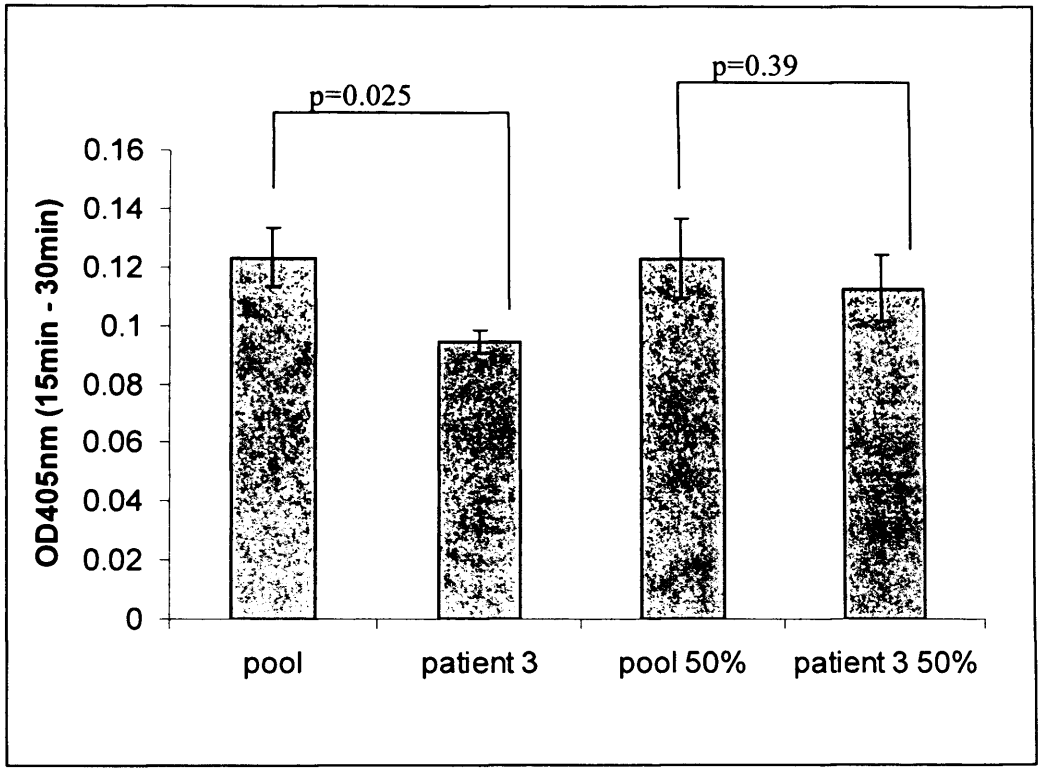


Figure 62 Difference in OD405nm of plate lysis curves for IgG fractions from pooled normal plasma and patient 3 at final concentrations of 0.36mg/ml and 0.18mg/ml.

The mean and standard deviation of triplicate wells in each arm are shown. P values are for an 2-tailed independent sample t-test.

To attempt to magnify any observed effect the IgG from patient 3 could be having in this system, I re-ran the experiment but with a reduced final concentration of uPA (0.1 μg/ml) and reduced thrombin concentration (0.57U/ml). Using these concentrations, it was possible to discern a greater difference in the aPL and non-aPL exposed arms of the experiment. The results of this experiment are shown in figure 63.

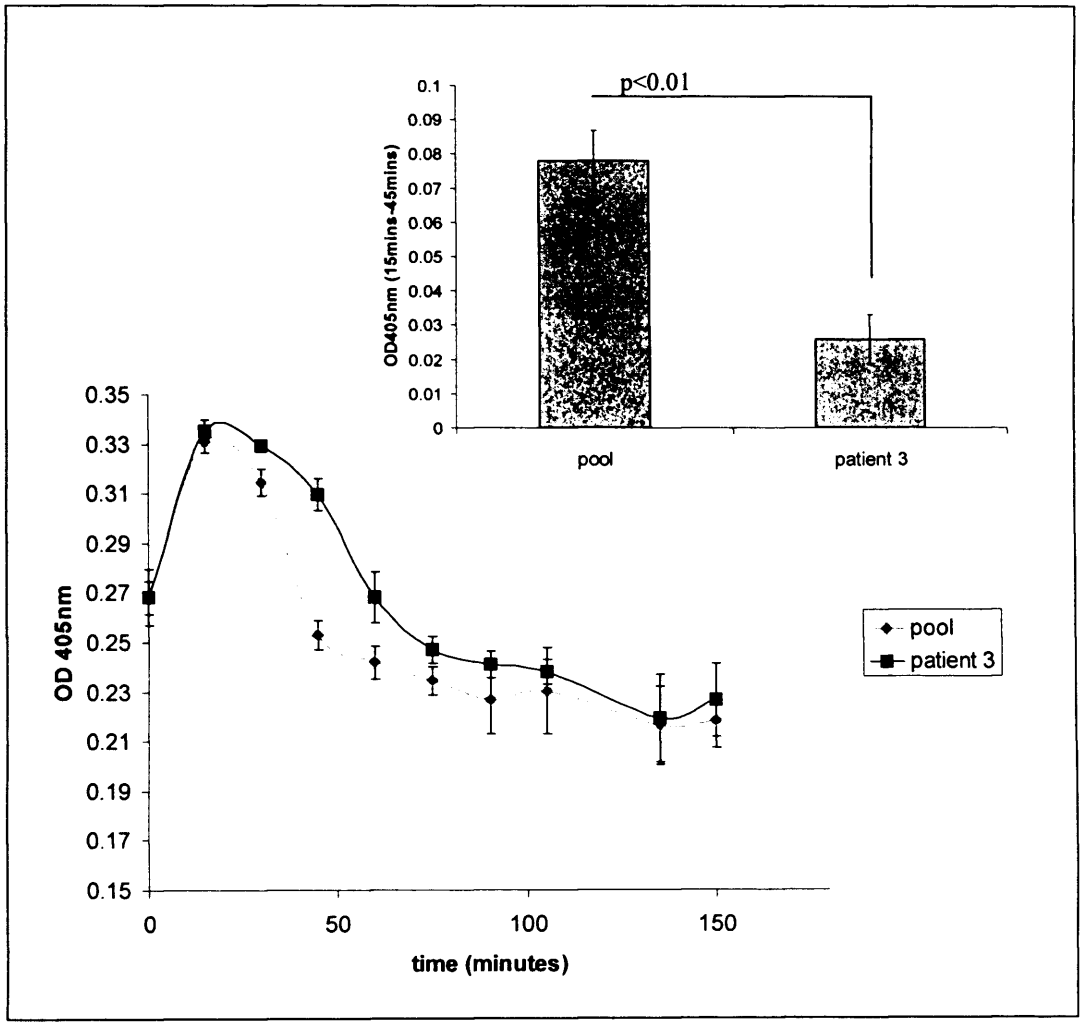


Figure 63 Plate lysis curve and difference in OD between the peak and 45 minute reading for pooled normal plasma and patient 3 IgG fractions.

Results show the mean and standard deviations for quadruplicate wells. The p value is for a 2 tailed independent samples t-test.

5.3.2 Further plate lysis experiments

The preceding experiments illustrated a consistent difference in plate lysis curves for the experimental arms using IgG fraction from patient 3. I was however not happy with the method I had used in these provisional experiments. In particular I was concerned with regard to the well loading method as it was employing small volume loads in the initial stages of the method and relied on the reagents being mixed in the wells of the 96 well plate. To this end having

attended a BSHT summer school on fibrinolytic methods, I modified the method used by Prof. Booth's team in Aberdeen University for microtitre plate clot lysis to allow spiking with aPL and pooled normal plasma IgG fractions. This method was then used to examine plate lysis in the presence of IgG fractions from seven patients with APS. This method has the advantage that the mixing of all the reagents is carried out in a test tube on ice and then the whole reagent mix is added to the 96 well plate rather than this being in separate steps as in the previous method.

Because this revised assay involved buffers containing calcium ions, I did not use phosphate buffers to avoid the potential precipitation of calcium phosphate. To this end, the IgG fractions used were exchanged on a de-salting column (PD-10, GE Healthcare) into the TRIS buffer described below.

For each experimental arm, two test tubes were set up. Tube 1 contained 200μl pooled normal plasma (Technoclone® diluted as per the manufacturer's instructions with water); 300μl IgG fraction (1mg/ml) from pooled normal plasma or a patient with APS in TRIS buffer; 80μl uPA (at 0.75 μg/ml in TRIS buffer). Tube 2 contained 200μl of bovine thrombin in normal saline (100u/ml); 300μl TRIS buffer; 500μl of 34mM $CaCl_2$. TRIS buffer was made up as follows: 10mM TRIS; 42.8mM Na Cl; 0.88mM KCl; pH 7.4. Reagents in each tube were mixed using a vortimixer and kept on ice. Eighty micro litres of tube 2 solution were added to tube 1, the solution mixed then 100μl of the resulting solution was added in quadruplicate to wells in a 96 well plate (96F microplate FX9200 Alpha laboratories). This process was carried out for a sample spiked with IgG from pooled normal plasma and one with IgG from a patient with APS. The optical

density was followed at 405nm every five minutes with the plate incubated at 37°C.

IgG fraction samples from patients 1,2,3,7,8,9 and 10 were examined in this way. As with the previous method, I found that there was too much inter-assay variation in lysis times (between the pooled normal plasma arms) to allow a group comparison of clot lysis times. I therefore once again compared the change in OD between the plateau phase of the curve and a time point roughly mid way in the pooled normal plasma IgG lysis curve. Results are shown in figures 64 to 66. A small difference in clot lysis was observed for samples spiked with IgG from patients 1,2,3,7 and 8 but not with samples from patients 9 or 10.

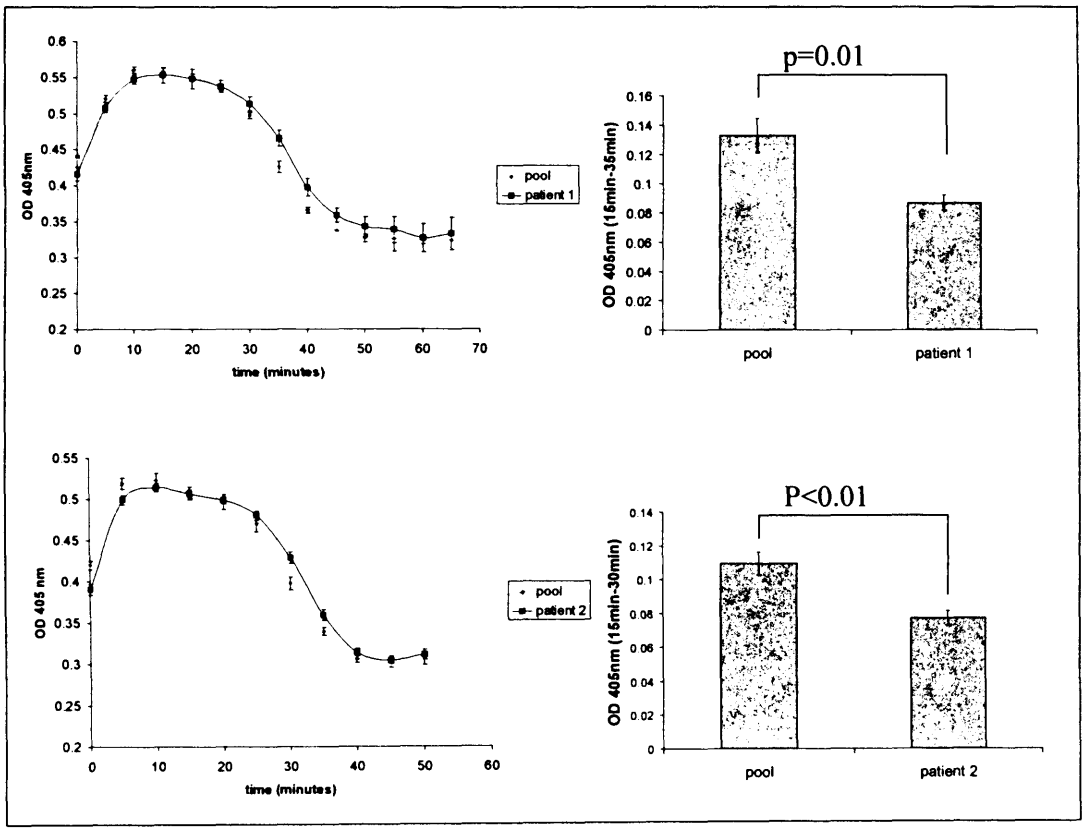


Figure 64 Clot lysis curves for patients 1 and 2 IgG fractions compared to pooled normal plasma IgG.

Results show mean and standard deviation for quadruplicate wells. P value for a 2 tailed independent samples t-test.

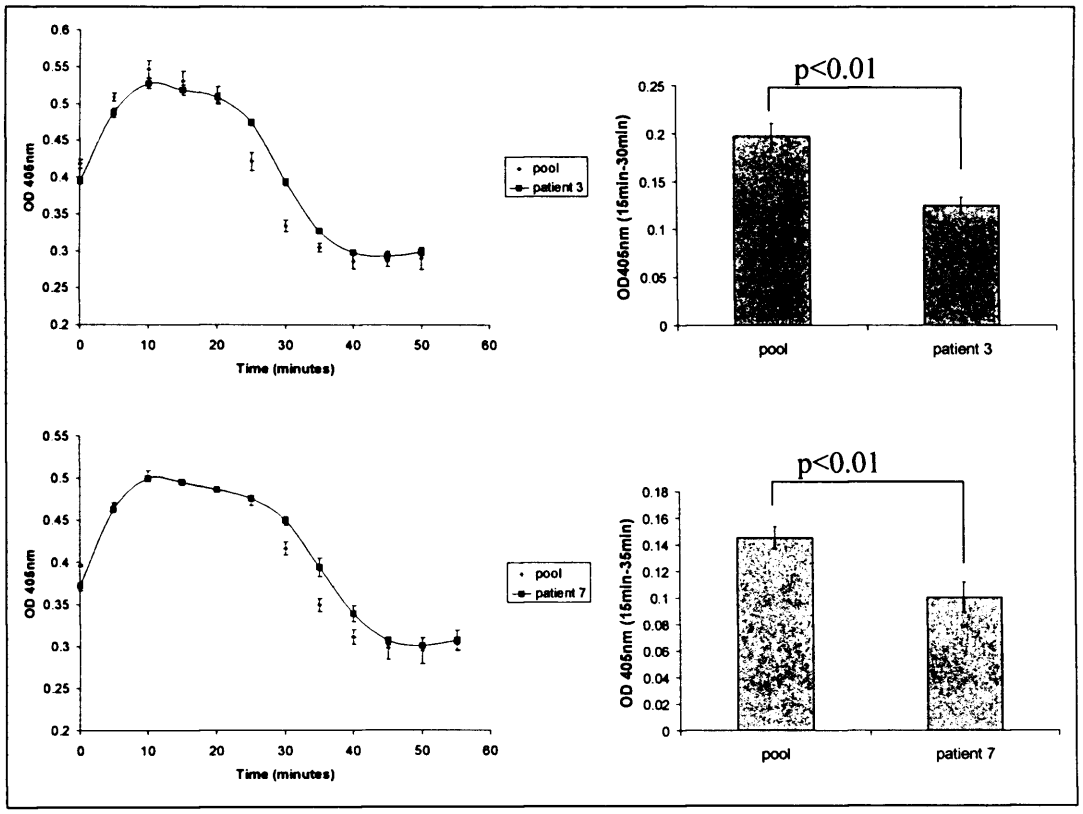


Figure 65 Clot lysis curves for patients 3 and 7 IgG fractions compared to pooled normal plasma IgG.

Results show mean and standard deviation for quadruplicate wells. P value for a 2 tailed independent samples t-test.

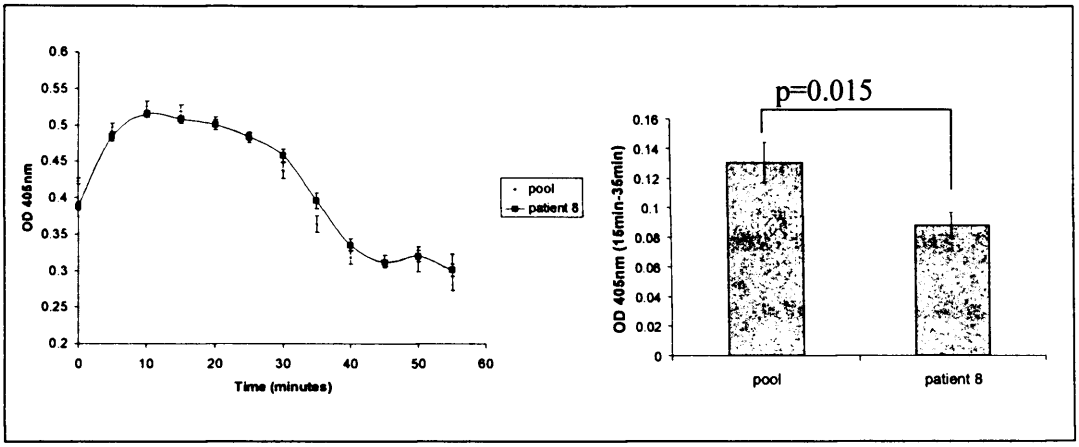


Figure 66 Clot lysis curves for patient 8 IgG fraction compared to pooled normal plasma IgG.

Results show mean and standard deviation for quadruplicate wells. P value for a 2 tailed independent samples t-test.

The results for the patient samples shown illustrate a small but significant difference in the degree of plasma clot lysis seen in this system. Again I found that the degree of intra-assay variation was too great to allow meaningful comparison of 50% clot lysis times between cases.

5.4 Discussion

5.4.1 The effect of aPL on the binding of plasminogen to HUVEC

The experimental work presented in this chapter demonstrates that purified IgG fractions from patients with aPL activity are in some cases able to reduce the binding of plasminogen to the surface of HUVEC. This effect was found in three patients initially examined using a flow cytometry system and in two of nine patients examined using a fixed cell monolayer system analysed using a fluorescent plate reader.

That all three of the patients examined initially showed an effect in the flow cytometry experiments is perhaps not surprising given their high aPL titres. It is however interesting that only one of them exhibited this effect when the experiment was repeated in a fixed cell monolayer system. There are several reasons why this may have occurred. As mentioned previously, the flow cytometer will assay binding of FITC-plasminogen to both the ex-luminal and basolateral surfaces of the HUVEC, while in the monolayer system only the luminal (i.e. upper) surface is assayed. It is possible and indeed probable that the binding characteristics for plasminogen of these two sites are different. Moreover, the pre-treatment of the cells in the flow cytometry system with Accutase to mobilise them involves exposure to the calcium chelating agent EDTA. The binding of Annexin II to cell surfaces is very calcium dependent and it is possible that surface Annexin II levels may have been reduced by this treatment. The monolayer experiments also involved fixing of the cells with PFA, and this may also have resulted in a change in binding characteristics between assays.

The magnitude of the reduction in plasminogen binding to the cell surface in those samples showing an effect is modest. Whether this would lead to a real reduction in "real" plasminogen activation and subsequent plasmin activity needs to be the subject of a subsequent project. With regards to the mechanism leading to the reduction in binding, the recent publication of anti-Annexin II activity in some patients with APS leading to functional impairment of the cell surface associated plasminogen activating system could provide an explanation for the effect I observed (Cesarman-Maus *et al*, 2006). Interestingly this group demonstrated anti-Annexin II activity in 22.6% of their APS cohort; although my numbers are small this is close to the proportion of samples demonstrating reduced plasminogen binding in the cell monolayer assay (22.2%). Further work in our cohort should be to determine whether anti-Annexin II activity correlated with the reduced plasminogen binding seen in the plasminogen binding assay.

Interestingly, the reduction in plasminogen binding observed does not seem to correlate with simple anti-endothelial cell surface activity. Most of the patient samples exhibited a significant increase in antibody binding to HUVEC, but not all showed plasminogen binding impairment activity. Again this implies a more specific interaction with a component of the cell surface – perhaps Annexin II. Our results here are similar to those of a recent paper also examining pericellular fibrinolysis in the presence of aPL (Patterson *et al*, 2006). This study demonstrated increased cellular binding of immunoglobulin from the sera of patients with APS in all fourteen cases examined. Again in spite of this, this study only revealed a functional consequence of this binding with respect to fibrinolytic activity in 4/14 cases examined (28.6%).

The work of Cesarman-Maus *et al* while illustrating activity against Annexin II and that this has functional implications does not demonstrate whether this is due to interference with binding of plasminogen or tPA (or both) to the HUVEC surface. While this body of work has demonstrated a reduction in plasminogen binding as possibly being important in any functional change noted, further work should examine the binding of tPA in the presence of aPL and perhaps the binding of Annexin II itself to the cell surface.

When activated by TNFα, the phenomenon of reduced plasminogen binding to the HUVEC surface continued to be present for the sample showing this effect in unstimulated cells. However, no further effect of cell activation was observed for those aPL samples not showing an effect on unactivated cells. I looked at the effect of cell activation on this system because of the recognised "two-hit" phenomenon observed in patients with APS. While inflammation itself is a risk for thrombosis, it does not appear that in APS the risk is further amplified by a reduction in endothelial cell plasminogen binding.

The work of Cesarman-Maus *et al* would seem to indicate that the binding of aPL to annexin II is independent of β2GPI. The HUVEC used in the experimental work in this chapter were incubated in media containing serum supplement and therefore are likely to have been exposed to β2GPI from this source. As discussed earlier, the source of β2GPI at the surface of EC has been debated in the literature (Caronti *et al*, 1999) (Alvarado-de la Barrera *et al*, 1998). An important future experiment to perform in the assay system used here would be to determine whether the effect seen in patients 3 and 7 is β2GPI dependent, perhaps by incubating the cells in a serum free media and repeating the experiments with and without an exogenous source of purified β2GPI.

While my work, and that of others has demonstrated that some patient aPL can affect the pericellular fibrinolytic system, this does not seem to be a mechanism seen in all patients with APS. As mentioned in the introduction to this thesis it may be that no one single mechanism accounts for the pathogenesis of APS and that various epiphenomena all contribute to the clinical picture seen in each patient with the syndrome.

5.4.2 The effect of aPL on the lysis of plasma clots in vitro

The second section of this chapter presents work illustrating the effect of aPL on the lysis of clots in a microtitre plate lysis system. A small effect on clot lysis was observed for five of seven aPL samples used in this experimental system. Previous work has demonstrated that IgG with aPL activity can cause this effect both for monoclonal antibody with aPL activity (Yang *et al*, 2004) and for purified IgG fraction from patients with APS (Kolev *et al*, 2002). The work of Yang *et al* utilised a direct application of plasmin to the clot surface while that of Kolev *et al* used tPA to activate plasminogen. In this study I have examined the effect of aPL on a clot lysis system activated by uPA.

Again this phenomenon is not one universally seen with the all aPL samples examined. That it was seen in a large proportion of the samples examined perhaps reflects the fact that patients with high titres of aPL were used in this study. The effect of the aPL containing IgG fractions is seen in samples from patients three and seven – samples which also exhibited reduced plasminogen cell surface binding in the monolayer experiments. It is also observed in samples negative in the cell plasminogen binding assay, suggesting a different mechanism is involved. This effect could be taking place at multiple levels in the fibrinolytic system. It has been suggested that aPL may reduce the

protective effect β2GPI exerts on plasminogen activators from PAI (Ieko *et al*, 2000). The effect could also be occurring at the level of the pattern of fibrin polymerisation in the presence of aPL or via a direct effect against plasminogen activators or plasmin itself. While antibody activity has been demonstrated against tPA in APS (Cugno *et al*, 2000), I am not aware of any study which has demonstrated anti-uPA activity in APS. A recent study in obstetric patients with APS has demonstrated a reduction in XIIa and functional uPA activity in comparison to the increase in activity usually seen with gestation; which the authors concluded was consequent upon reduced XII activation resulting in reduced uPA activity (Carmona *et al*, 2006). To date no study has demonstrated an effect of aPL in a clot lysis system activated by uPA as presented here.

Whether global reduction in fibrinolytic potential represents a risk for thrombosis has been addressed by a recent study by Lisman *et al* (Lisman *et al*, 2005). This study (of the Leiden Thrombophilia Study Cohort) found using a tPA activated clot lysis assay, that hypofibrinolysis was associated with a dose dependent increase in risk of DVT. Although not directly comparable, this would indicate that an observed reduction in fibrinolysis in the presence of aPL may have some clinical relevance.

Chapter 6

Analysis of the Cys306Gly polymorphism of β2-glycoprotein I in relation to obstetric and thrombotic complications and aPL positivity

6.1 Introduction

Stable polymorphisms of the β2GPI gene exist in the human genome. Two of these the Trp316Ser and the Cys306Gly polymorphisms result in significant changes to the structure of domain V of β2GPI, such that its binding to negatively charged phospholipid surfaces is impaired. While undertaking the thesis, I became interested in these polymorphisms and as discussed in earlier chapters examined their susceptibility to be plasmin cleaved. Their impaired phospholipid binding was also important in my development of a cardiolipin binding assay which was used in the work in chapter 4.

Our research group had previously studied the relationship between the Trp316Ser and Val247Leu polymorphisms of β2GPI in relation to aPL positivity and obstetric/thrombotic pathology with largely negative results (Camilleri *et al*, 2003). Given the large effect the Cys306Gly polymorphism has on β2GPI structure and function, I undertook to examine the frequency of this polymorphism in our patient cohort. In the first instance I was interested in whether there was a difference in the incidence of aPL between individuals with the polymorphism and wild type, specifically as to whether the presence of the polymorphism protected against the production of aPL activity. This work also

identified the individual who was a compound heterozygote for both domain V polymorphisms whose samples were used in chapter 4.

6.1.1 Background

6.1.1.1 β2 Glycoprotein I polymorphisms

Seven stable polymorphisms resulting in amino acid sequence changes have been described for β2GPI. In addition, a polymorphism in the transcription initiation site of the β2GPI gene, which results in lower protein expression, has also been described (Mehdi *et al*, 2003). A missense mutation at codon 88 results in a Ser to Asn substitution while another at codon 316 leads to a Trp to Ser substitution (Sanghera *et al*, 1997a). The mutation affecting codon 316 affects the phospholipid binding capacity of the protein because a hydrophilic residue becomes present in the hydrophobic loop of domain 5 (Sanghera *et al*, 1997b). A missense mutation at codon 247 results in a Val to Leu substitution (Steinkasserer *et al*, 1993). As well as the 316 polymorphism, another missense mutation which results in diminished phospholipid binding of β2GPI has been described – at codon 306 (Sanghera *et al*, 1997b). This polymorphism (TGC to GGC) results in a Cys to Gly substitution leading to disruption of the disulphide bond between Cys306 and 281, which is crucial for the maintenance of the structure of domain V. Previous studies have looked at the influence of the 306 polymorphism on the production of aPL and found no effect. However, the cohorts in these studies consisted predominantly of patients with systemic lupus erythematosus and/or secondary APS (Gushiken *et al*, 1999) (Kamboh *et al*, 1999).

Structural variants of β2GPI were first described by means of isoelectric focusing and immunoblotting. Under this classification (in which β2GPI was still referred to as apolipoprotein H), the most common variant was APOH*2 followed by APOH*1 and 3. A fourth variant, APOH*4, has been observed only in populations of African descent. APOH*3 was further categorised into $*3^{W}$ and $*3^{B}$ isoforms based on reactivity to the monoclonal antibody 3D11 (Kamboh *et al*, 1995). The molecular basis of some of these isoelectric focusing (IEF) variants has been determined. APOH*1 corresponds to the codon 88 polymorphism and APOH$*3^{W}$ to the 316 polymorphism (Sanghera *et al*, 1997a). A more recent study by the Kamboh group (published after the experimental work for this thesis had been carried out) has further explored the nature of the *1-*4 variants. Three further polymorphisms have been described in the β2GPI gene at positions 122, 135 and 141 (Ile122Thr; Arg135His; Ala141Asp). The APOH$*3^{B}$ allele expresses the 122 and 135 polymorphisms, this form of β2GPI corresponds to the Chimpanzee wild type and may be the prototype ancestral β2GPI gene. The $*3^{W}$ form has the 122 and 135 polymorphisms along with the Trp316Ser polymorphism. The APOH*2 allele – the human wild type, lacks the 122 and 135 polymorphisms as well as being negative for the 88, 247, 306 and 316 polymorphisms. The APOH*4 gene is positive for the 122 and 135 polymorphisms as well as an additional polymorphism at position 141 (Kamboh *et al*, 2004). This group has also presented biochemical data showing reduced binding of β2GPI with the 141 polymorphism and with a combination of the 122 and 135 polymorphisms as exhibiting reduced binding to cardiolipin (around 30% reduction in each case). These results are interesting in that they reveal changes in domain III of β2GPI affecting binding to phospholipid.

6.1.1.2 Trp316Ser and Cys306Gly polymorphisms

Of all the polymorphisms described for β2GPI those at codons 306 and 316 to date have been shown to have the most profound effects on the structure and function of the protein. Both of these amino acid changes result in disruption of the region of β2GPI crucial for binding to phospholipid. The 306 polymorphism results in disruption of a disulphide bond crucial to the structure of domain V while the 316 polymorphism replaces a hydrophobic for a hydrophilic amino acid in the hydrophobic loop of domain V. Soon after their discovery, demonstration of their reduced binding to negatively charged phospholipid was published (Sanghera *et al*, 1997b). The 306 and 316 polymorphisms have also been demonstrated to result in lower plasma β2GPI levels (Mehdi *et al*, 1999) (Kamboh *et al*, 1999). These two polymorphisms may also act in concert meaning a compound heterozygote for the 306 and 316 polymorphisms would exhibit reduced binding of β2GPI to phospholipid. The functional significance of heterozygosity for the 306 or 316 polymorphisms alone could be questioned. The anionic phospholipid binding of β2GPI from individuals heterozygous for the 306 or 316 polymorphisms has been examined and found to be normal (Sanghera *et al*, 1997b) (Gushiken *et al*, 2002). It has been suggested that this effect is due to compensation by wild type β2GPI which can compensate for reduced binding of the variant form because β2GPI circulates in a multimeric form.

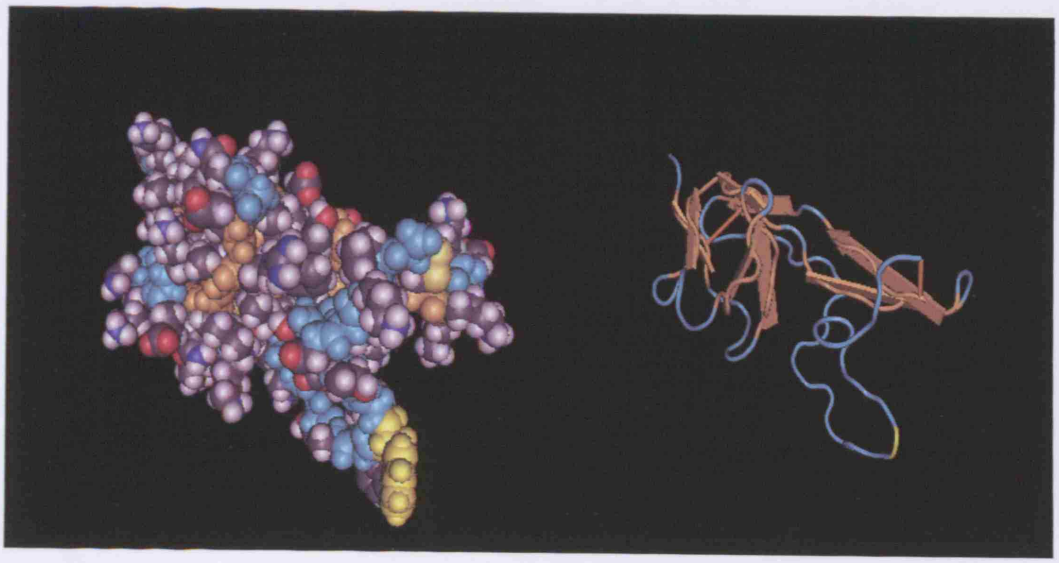


Figure 67 The Trp316Ser polymorphism of β2GPI

Space filling and worm illustrations of the structure of domain V of β2GPI. The Trp316 residue is highlighted in yellow. The worm structure on the right illustrates the hydrophobic loop crucial to the protein-phospholipid interaction. (Structure diagram generated at the Entrez-structure website).

These changes in the biochemistry of β2GPI, arising from sequence changes at codons 306 and 316, have led workers to examine their effect on the incidence of APL production. Kamboh *et al* (Kamboh *et al*, 1999) examined a group of SLE patients and found the incidence of the 316 polymorphism was significantly lower in patients with aPL suggesting its presence may have some protective effect impeding the antigenicity of β2GPI. This study did not find a similar effect in relation to the presence of the 306 polymorphism. An earlier study (Horbach *et al*, 1998) of SLE patients had failed to show any relationship between the 316 polymorphism and aPL production. Kamboh *et al,* however, pointed out that the earlier study had used a mixed race population in their analysis while their study had limited itself to Caucasian subjects only. Notably,

the population in the study by Kamboh *et* al was screened only once for the presence of aPL. No association between the 316 polymorphism and aPL positivity was found in a study performed in our department at UCLH (Camilleri *et al*, 2003). A study examining the relationship of the 306 and 316 polymorphisms with aPL production in a population of SLE patients failed to show any association. Interestingly, this study raised the possibility of a weak association between the presence of the 316 polymorphism and the occurrence of thrombosis (Gushiken *et al*, 1999).

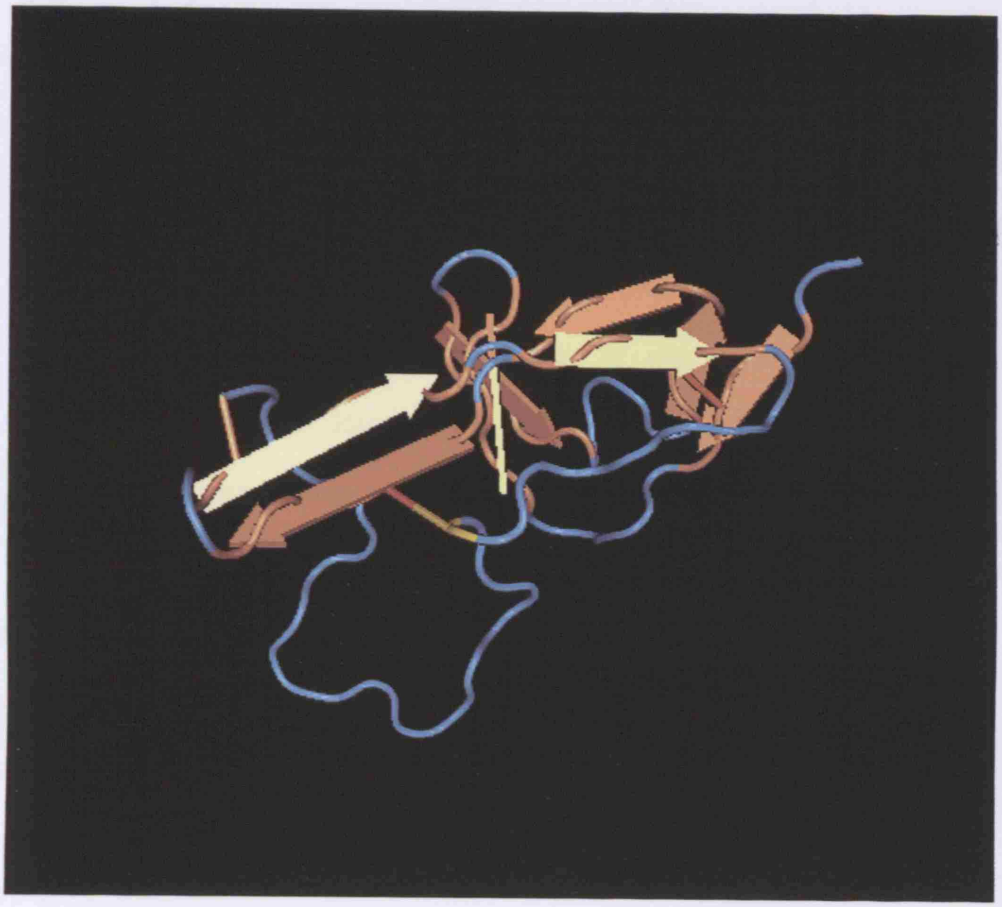


Figure 68 The Cys306Gly polymorphism of β2GPI

A worm diagram of domain V of β2GPI. The position of Cys306 is highlighted in yellow. It can be seem that the disulphide bond it forms with Cys281 is crucial for the integrity of the phospholipid interacting apparatus of domain V. (Structure diagram generated at the Entrez-structure website).

6.1.1.3 Val247Leu polymorphism

This stable polymorphism of the β2GPI gene was first described in 1993 (Steinkasserer *et al*, 1993); interestingly, this variant does not correspond to one of the four isoelectric focusing alleles of β2GPI. In contrast to the 306 and 316 polymorphisms, the 247 polymorphism does not affect plasma levels of β2GPI (Kamboh *et al*, 1999). Kamboh *et al* also did not report any association between the 247 polymorphism and the occurrence of APL in their cohort of SLE

patients. This finding contrasts with the results of Atsumi *et al* (Atsumi *et al*, 1999), who found the Val247 allele to be more frequent in patients with primary APS with or without anti-β2GPI activity compared to controls. Like Kamboh *et al*, this study found no change in allele frequency when patients with secondary APS were considered. A study in a paediatric population has also described an association between a Val at position 247 and aPL production (von Scheven & Elder, 2005). A recent study in a Japanese population has also shown a correlation between the Val247 polymorphism and aPL production. In addition, this group demonstrated increased binding of aPL to the Val247 variant compared to the Leu247 form. The group went on to describe modelling data suggesting that substitution of Leu for Val at position 247 results in tertiary structure changes in domain V of β2GPI via domain IV-V interaction (Yasuda *et al*, 2005)

6.2Methods

6.2.1 Patients and control groups

Two-hundred and ninety consecutive patients referred to our haemostasis clinic were studied. The group consisted of 209 Caucasians, 24 Afro-Caribbean individuals, 30 Asians and 27 individuals of other ethnic origins. Patients presented with the following conditions: 165 thrombosis (arterial and/or venous), 55 late pregnancy morbidity (pre-eclampsia), 86 repeated/recurrent miscarriage, 11 miscellaneous (1 Bechet's Disease; 1 TTP; 4 migraine; 2 thrombosis family history; 1 small vessel disease; 2 SLE) (note some patients had more than one clinical presentation). Three of the patients in the study were documented as having SLE. Two hundred and twenty-two of the patients were female and 68 male. The mean patient age was 41.8 years. The control group consisted of 284 healthy individuals with no previous history of thrombotic or obstetric disorder (215 female and 69 male, mean age 62.5 years).

Antiphospholipid antibody testing for IgG and IgM anticardiolipin antibodies, IgG and IgM anti-β2GPI antibodies and lupus anticoagulant activity was carried out as described in the general methods section.

6.2.2 DNA extraction

DNA was extracted from buffy coat samples of patients and controls using the "quick prep" method from the UCLH haematology laboratory standard operating procedure. 100μl of well-mixed buffy coat was added to a 1.5ml microcentrifuge tube and 800μl of ammonium chloride solution (0.091g/10ml) was added. The solution was mixed by inversion for 50 minutes and then

centrifuged (10,000g) for 2 minutes. The supernatant was discarded and the pellet washed in 1ml of 0.85% saline, vortexed and re-centrifuged as above. 200µl of NaOH (0.05M) was then added, vortexed and heated to 99°C for 10 minutes (using a thermal cycler), pH was then neutralised by addition of 40µl 1M TRIS buffer (pH 7.5-8) the sample vortexed and stored at -20°C.

6.2.3 Polymerase chain reaction and restriction digest to detect the Cys306Gly polymorphism.

The Cys306Gly polymorphism of β2GPI was detected using a combination of PCR to amplify exon 7 of the β2GPI gene and restriction enzyme-digest with the enzyme *StuI*.

Primer sequences and the rationale of the method used are illustrated in figures 69 and 70. The reverse primer RAYAPOH14B contained a sequence change designed on amplification with PCR to create a restriction enzyme site for StuI in the presence of the sequence change associated with the Cys306Gly polymorphism. This method of restriction digest site production is sometimes referred to as the Amplification Created Restriction Enzyme Site method or ACRES.

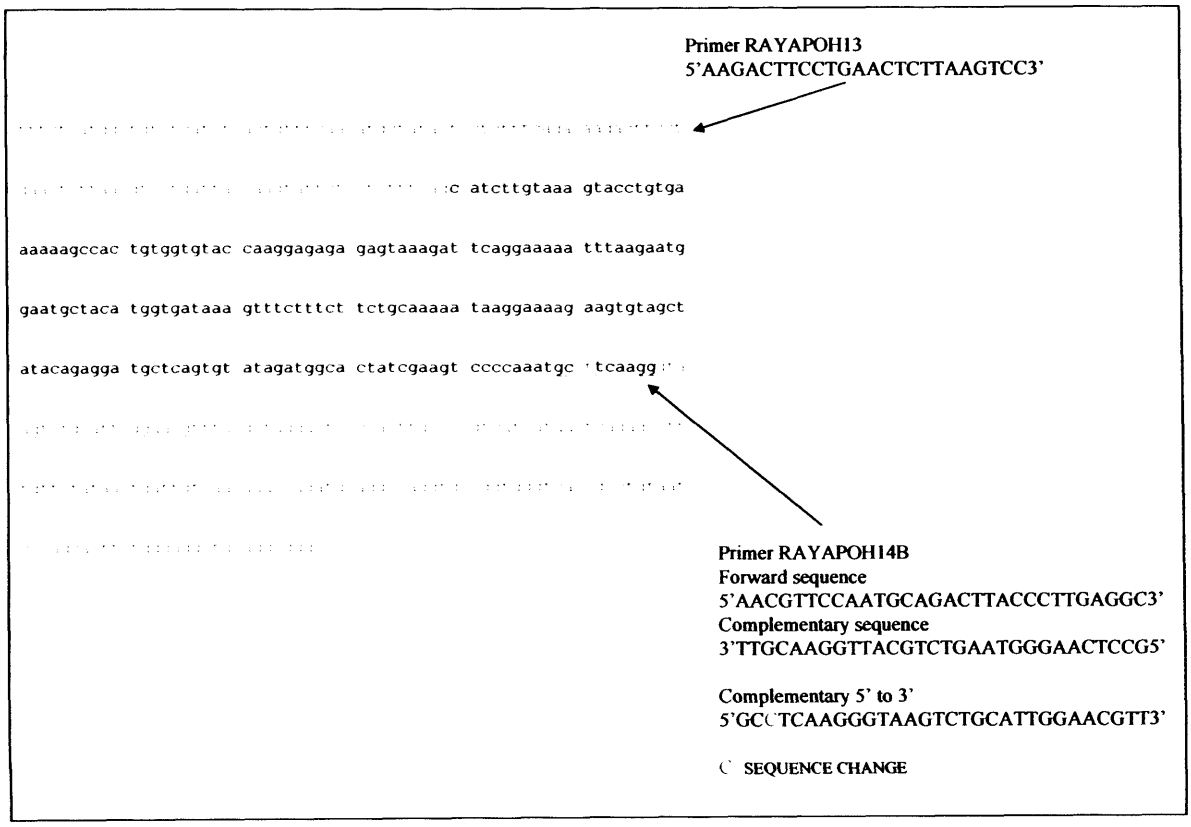


Figure 69 Primer design for amplification of exon 7 of β2GPI

Intronic sequences are shown in blue, exon 7 in black. The sequence change in primer RAYAPOH14B is shown in red.

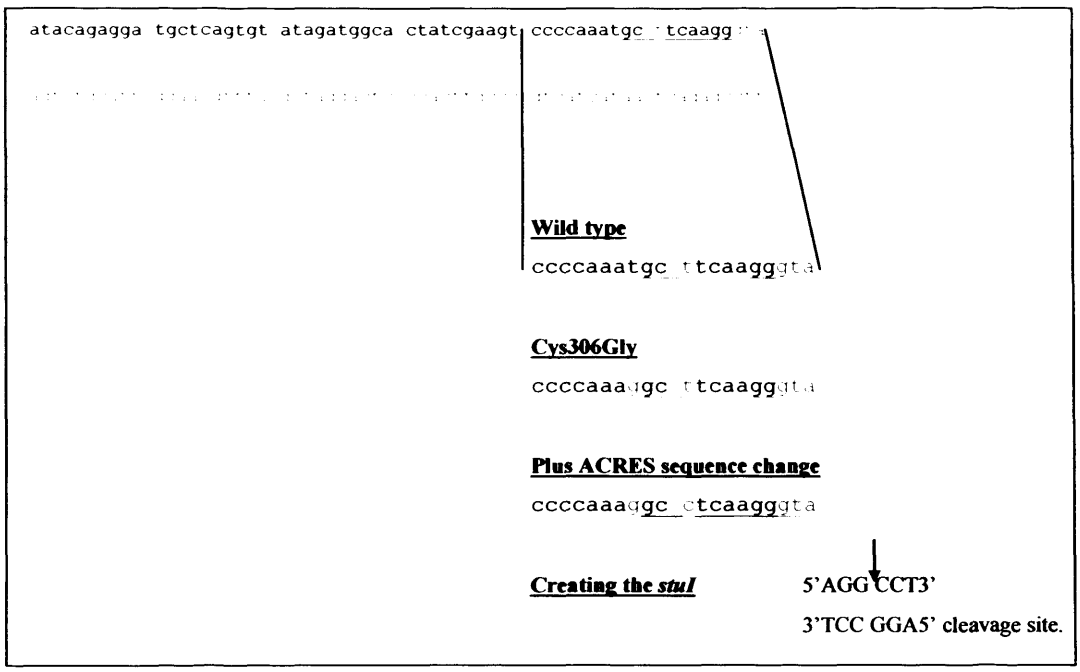


Figure 70 Illustration of the creation of a *Stu I* restriction digest site in the presence of the Cys306Gly polymorphism.

For each PCR reaction, the following reaction tube was set up: 10µl DNA template; 2.5µl NH_4 buffer (Bioline); 0.5µl deoxy trinucleotide phosphates (10mM, Promega); 1.5µl 25mM $MgCl_2$; 1µl of each primer (25pico moles each); 7.5µl sterile water. In each run, a control reaction containing sterile water instead of DNA was included. Reaction contents were sealed with one drop of mineral oil. Tubes were heated on a thermal cycler to 95°C for 5 minutes and then had 1µl of Taq polymerase added (1/10 diluted in sterile water, Bioline). The PCR tubes were then cycled 30 times as follows: 95°C for 30 seconds, 62°C for 30 seconds, 72°C for one minute. This was followed by 72°C for 5 minutes (1 cycle). An aliquot of each reaction product was then visualised via agarose gel (2-4%) electrophoresis and ethidium bromide staining using a UV transilluminator.

Restriction digestion of the PCR product was carried out at 37°C for 18 hours using *Stu*I in NE Buffer 2 (New England Biolabs) as per the instructions of the manufacturer. The digest product was visualised as described above. Two distinct bands were easily visualised in the presence of heterozygosity for the Cys306Gly polymorphism. A single lower molecular weight band was seen in the presence of homozygosity for the polymorphism. Representative reaction products are shown in figure 71.

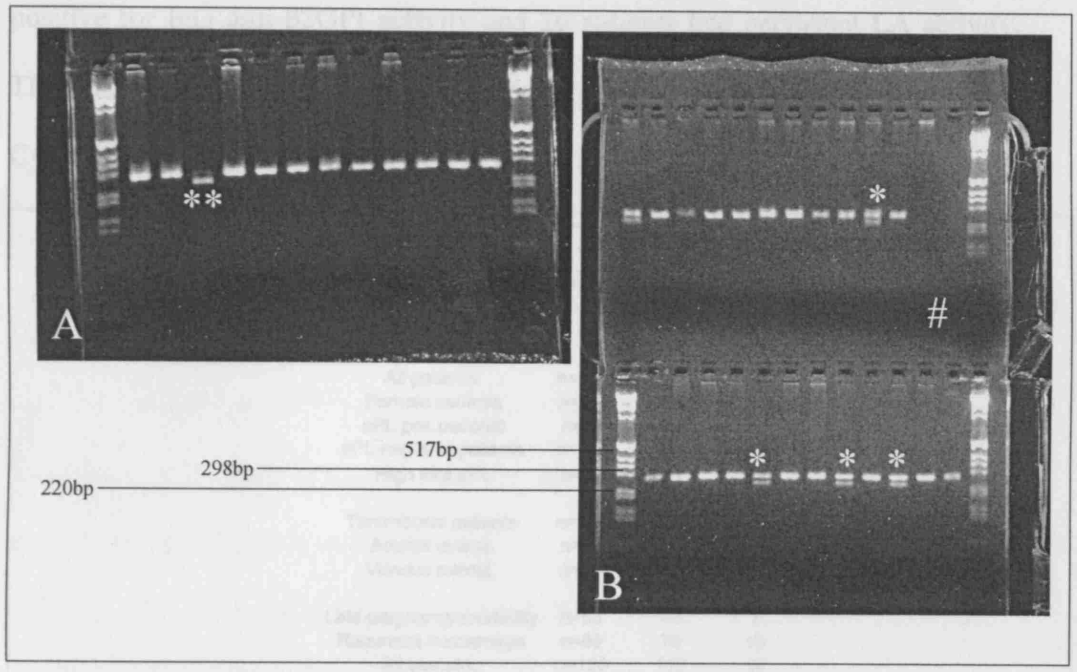


Figure 71 Restriction digest (*Stu*I) PCR products of exon 7 of β2GPI.

(A) A homozygous patient for the Cys306Gly polymorphism labelled **. (Because of lack of clinical details, this patient was not included in the study). (B) Heterozygous patients labelled *. Molecular weight markers confirm the expected position of the 268bp reaction product. # indicates the position of two control lanes (no DNA added to PCR reaction tubes).

6.2.4 Statistical analysis

Odds ratios with 95% confidence intervals were calculated and Fisher's exact test used to compare genotype frequencies between patient and control groups. An initial analysis was performed on all individuals in the test and control groups then a second analysis was limited to Caucasian individuals from

each group only. A p value of less than 0.05 was taken as significant. The STATA 7 statistics package was used in the analysis (Stata Corporation, College station, Texas, USA; distributed by Timberlake consultants LTD. London).

6.3 Results

Of the 290 patients studied, 97 were found to be persistently positive for aPL. Sixty-four of the aPL positive patients were persistently positive for IgG aCL antibodies, 33 were persistently positive for IgM aCL, 32 were persistently positive for IgG anti-β2GPI activity and 26 patients had persistent LA activity. The distributions of the patients between various clinical subgroups and their Cys306Gly genotype are illustrated in tables 23 and 24.

Group		Genotype	
		T/T	T/G
All controls	n=284	268	16
Female controls	n=215	203	12
All patients	n=290	261	29
Female patients	n=222	199	23
aPL pos patients	n=97	88	9
aPL negative patients	n=193	173	20
High titre aPL	n=38	35	3
Thrombosis patients	n=165	150	15
Arterial events	n=85	74	11
Venous events	n=89	85	4
Late pregnancy morbidity	n=55	48	7
Recurrent miscarriage	n=86	76	10
All obstetric	n=126	112	14

Group		Genotype	
aPL positive patients		T/T	T/G
Thrombosis patients	n=61	54	7
Arterial events	n=33	27	6
Venous events	n=29	28	1
Late pregnancy morbidity	n=12	10	2
Recurrent miscarriage	n=18	18	0
All obstetric	n=28	26	2

Group		Genotype	
aPL negative patients		T/T	T/G
Thrombosis patients	n=104	96	8
Arterial events	n=52	47	5
Venous events	n=60	57	3
Late pregnancy morbidity	n=43	38	5
Recurrent miscarriage	n=68	58	10
All obstetric	n=98	86	12

Table 23 Distribution of all patients between clinical groups and genotype distribution.

T/T=wild type codon 306 T/G=Cys306Gly polymorphism heterozygote. No homozygous patients were included in the study.

Group		Genotype	
		T/T	T/G
All controls	n=274	258	16
Female controls	n=205	193	12
All patients n=290	n=209	186	23
Female patients	n=159	141	18
aPL pos patients	n=68	61	7
aPL negative patients	n=141	125	16
High titre aPL	n=25	23	2
Thrombosis patients	n=131	117	14
Arterial events	n=66	56	10
Venous events	n=73	69	4
Late pregnancy morbidity	n=34	29	5
Recurrent miscarriage	n=52	46	6
All obstetric	n=78	69	9

Group		Genotype	
aPL positive patients		T/T	T/G
Thrombosis patients	n=49	43	6
Arterial events	n=26	21	5
Venous events	n=24	23	1
Late pregnancy morbidity	n=8	7	1
Recurrent miscarriage	n=9	9	0
All obstetric	n=15	14	1

Group		Genotype	
aPL negative patients		T/T	T/G
Thrombosis patients	n=82	74	8
Arterial events	n=40	35	5
Venous events	n=49	46	3
Late pregnancy morbidity	n=26	22	4
Recurrent miscarriage	n=43	37	6
All obstetric	n=63	55	8

Table 24 Distribution of Caucasian patients between clinical subgroups and genotype distribution

T/T=wild type codon 306 T/G=Cys306Gly polymorphism. No homozygous patients were included in the study.

Analysis of genotype distribution between groups did not reveal any significant difference between controls and patients with persistent aPL with regard to the Cys306Gly polymorphism. This finding was consistent when patients with high titre aPL (aCL>40 GPLU/MPLU and/or anti-β2GPI>30% and/or persistently LA positive) were considered as a separate group.

A consistent finding across the whole patient group and the Caucasian only group was a significant increase in frequency of the Cys306Gly polymorphism in patients with arterial events. These patients in the main had been referred to our clinic for further investigation of ischaemic stroke; twelve of the patients in this group had suffered transient ischaemic attacks. Across the whole sample group arterial events were associated with the Cys306Gly polymorphism with an odds ratio of 2.49 (95%CI 1.00-5.98), this rose to an odds

ratio of 2.88 (95%CI 1.10-7.15) in the Caucasian only group. In both sample analyses, a significant increase in the frequency of the Cys306Gly polymorphism was seen in aPL positive patients with arterial symptoms, but not in aPL negative patients when they were analyzed as a subgroup. The results for arterial event patients remained significant when allele frequencies were also considered (data not shown). Within the arterial events group, 68 of the patients had suffered an ischaemic stroke, 4 a myocardial infarction, 3 lower limb ischaemia and 12 episodes of transient ischaemic attack. One of the arterial event patients had been diagnosed with SLE and eight had also suffered a venous thrombotic event.

Unfortunately, because this study was originally set up to examine an association between the production of aPL and the Cys306Gly polymorphism and not one with arterial pathology, no data was collated on the general cardiovascular risk factors in the patients examined. To further explore any association, another study will need to be performed in which such data is analyzed in conjunction with β2GPI genotype.

In the non-selected patient group a significant association was seen between recurrent miscarriage in aPL negative patients and the Cys306Gly polymorphism. This failed to maintain significance when only Caucasian patients were looked at.

A summary of the p values (by Fisher's exact test), odds ratios and 95% confidence intervals for each of the group comparisons made is shown in table 25 and 26.

Comparison (2 tailed Fisher's exact test)	p value	OR	95%CI
All patients vs all controls	0.06	1.86	0.95-3.76
Female patients vs female controls	0.07	1.96	0.90-4.43
aPL positive patients vs all controls	0.24	1.71	0.64-4.29
aPL negative patients vs all controls	0.07	1.94	0.92-4.11
aPL positive vs aPL negative patients	0.83	0.88	0.34-2.14
High titre aPL patients vs all controls	0.47	1.44	0.26-5.38
Thrombosis patients vs all controls	0.18	1.68	0.75-3.73
Arterial event patients vs all controls	**0.03**	**2.49**	**1.00-5.98**
Venous event patients vs all controls	0.79	0.79	0.19-2.54
LP patients vs female controls	0.08	2.46	0.78-7.20
RM patients vs female controls	0.09	2.23	0.82-5.87
All obstetric patients vs female controls	0.08	2.11	0.87-5.18

APA positive patients

Comparison (2 tailed Fisher's exact test)	p value	OR	95%CI
Thrombosis patients vs all controls	0.15	2.17	0.72-5.90
Arterial event patients vs all controls	**0.02**	**3.72**	**1.09-11.04**
Venous event patients vs all controls	1	0.6	0.01-4.15
LP patients vs female controls	0.16	3.38	0.32-18.63
RM patients vs female controls	0.6	0	0-4.46
All obstetric patients vs female controls	0.67	1.3	0.13-6.35

APA negative patients

Comparison (2 tailed Fisher's exact test)	p value	OR	95%CI
Thrombosis patients vs all controls	0.47	1.4	0.50-3.59
Arterial event patients vs all controls	0.34	1.78	0.49-5.40
Venous event patients vs all controls	1	0.88	0.16-3.23
LP patients vs female controls	0.17	2.23	0.58-7.26
RM patients vs female controls	**0.02**	**2.92**	**1.07-7.76**
All obstetric patients vs female controls	0.06	2.36	0.93-5.98

Table 25 Results of statistical analysis between all patients and control groups for genotype distribution of the Cys306Gly β2GPI polymorphism.

OR=odds ratio; 95%CI=95% confidence interval. LP=late pregnancy morbidity; RM=recurrent miscarriage. Significant p values, OR and 95% CI shown in bold.

Comparison (2 tailed Fisher's exact test)	p value	OR	95%CI
All patients vs all controls	**0.04**	**1.99**	**0.98-4.15**
Female patients vs female controls	0.08	2.05	0.9-4.83
aPL positive patients vs all controls	0.18	1.85	0.61-5.00
aPL negative patients vs all controls	0.053	2.06	0.93-4.56
aPL positive vs aPL negative patients	1	0.9	0.30-2.45
High titre aPL patients vs all controls	0.65	1.4	0.15-6.58
Thrombosis patients vs all controls	0.1	1.93	0.84-4.37
Arterial event patients vs all controls	**0.02**	**2.88**	**1.10-7.15**
Venous event patients vs all controls	1	0.93	0.22-3.03
LP patients vs female controls	0.07	2.77	0.71-9.20
RM patients vs female controls	0.21	2.1	0.61-6.42
All obstetric patients vs female controls	0.13	2.1	0.74-5.68

APA positive patients

Comparison (2 tailed Fisher's exact test)	p value	OR	95%CI
Thrombosis patients vs all controls	0.12	2.25	0.68-6.46
Arterial event patients vs all controls	**0.03**	**3.84**	**0.99-12.37**
Venous event patients vs all controls	1	0.7	0.02-4.94
LP patients vs female controls	0.4	2.3	0.05-20.39
RM patients vs female controls	1	0	0-9.27
All obstetric patients vs female controls	1	1.15	0.03-8.91

APA negative patients

Comparison (2 tailed Fisher's exact test)	p value	OR	95%CI
Thrombosis patients vs all controls	0.22	1.74	0.62-4.52
Arterial event patients vs all controls	0.16	2.3	0.62-7.10
Venous event patients vs all controls	1	1.05	0.19-3.89
LP patients vs female controls	0.08	2.92	0.63-10.72
RM patients vs female controls	0.09	2.61	0.75-8.06
All obstetric patients vs female controls	0.09	2.34	0.78-6.56

Table 26 Results of statistical analysis between Caucasian patient and control groups for genotype distribution of the Cys306Gly β2GPI polymorphism.

OR=odds ratio; 95%CI=95% confidence interval. LP=late pregnancy morbidity; RM=recurrent miscarriage. Significant p values, OR and 95% CI shown in bold.

6.4 Discussion

The results presented in this section are in agreement with previous studies which have shown that the presence of the Cys306Gly polymorphism does not influence the production of aPL (Gushiken *et al*, 1999) (Kamboh *et al*, 1999). This finding was consistent when only high-titre aPL patients were considered as a separate group.

The data on this cohort of patients and control subjects shows a comparable frequency for the 306 polymorphism in control subjects compared to previous papers (5.6% in all controls and 5.8% in Caucasian controls). Any differences may reflect the different geographical locations of the studies. With regard to the patient populations examined, in the case of Gushiken *et al,* most of the patients examined had SLE or connective tissue disease; in the case of Kamboh *et al,* all the patients had SLE. This is in contrast to our mainly SLE negative population. This reflects the case distribution at UCLH where most of the SLE patients are under the care of the rheumatology clinic and not the haematology clinic, from which the patients in this study were recruited.

With regard to the frequency of the Cys306Gly polymorphism between Caucasian and non-Caucasian patient populations no significant difference was observed ($p=0.5$, 2-tailed Fisher's exact test). No analysis of this nature was performed on the control population given the small number on non-Caucasians in this group.

With respect to the comparisons made in this study between patient and control groups (and aPL positive and negative groups) which failed to show a

significant difference the possibility of a type II statistical error exists in all cases due to the small number of cases examined.

In the patients examined with arterial pathology, a significant increase in the frequency of the Cys306Gly polymorphism was found. A previous study has indicated that an association may exist between a Val at position 247 of β2GPI and arterial thrombosis (Prieto *et al*, 2003). Moreover, a trend towards an association between thrombosis and the presence of the Trp316Ser β2GPI polymorphism has been described (Kamboh *et al*, 1999). Given the lack of association between aPL positivity and the Cys306Gly polymorphism found in this study, an alternative explanation from aPL production needs to be sought to explain this finding. That no previous study has shown this association is perhaps reflective of the differences in the populations studied, as discussed above. Unfortunately no data was acquired during this study to enable a test to be performed on whether the presence of the Cys306Gly polymorphism is an independent cardiovascular risk factor; a future study should aim to do this.

One possible explanation for these findings could relate to the lower levels of β2GPI seen in patients with the Cys306Gly polymorphism (Kamboh *et al*, 1999). Lower β2GPI levels are associated with reduced cholesterol, HDL cholesterol and triglyceride levels. Any disproportionate reduction in HDL levels in relation to LDL could result in adverse arterial event risk. In order to test this hypothesis, it would be necessary to test fasting lipid levels in our patient and control groups. With this hypothesis in mind, however, one should look back at the results of Yasuda *et al* who failed to demonstrate any difference in lipid profile between controls and individuals with β2GPI deficiency (Yasuda *et al*, 2000). Given some of the anticoagulant functions of β2GPI (discussed in the

introduction to this thesis), one could speculate that reduced levels of β2GPI could mediate a pro-coagulant effect by means of reduction in these activities. Again, however, studies on β2GPI deficient individuals have failed to show any significant procoagulant changes (Takeuchi *et al*, 2000). Another potential level of interaction for the Cys306Gly polymorphism and arterial pathology lies at the level of the β2GPI-oxidised LDL interaction. The presence of β2GPI has been shown to impede oxidised LDL uptake by macrophages (thought to be an important step in atherogenesis) (Hasunuma *et al*, 1997). Perhaps the presence of the Cys306Gly polymorphism impairs this interaction even at the level of β2GPI multimers. Further experimental work is needed to explore this possibility.

A potentially novel level of interaction for the Cys306Gly polymorphic variant exists at the level of fibrinolysis. As discussed earlier in this thesis, clipped β2GPI with a disrupted domain V can inhibit fibrinolysis (Yasuda *et al*, 2004). It would be interesting to see whether β2GPI with a disrupted domain V secondary to the Cys306Gly polymorphism could exert a similar effect as clipped β2GPI. Given the observations of a trend towards increased thrombosis in patients with the Trp316Ser polymorphism and the increased incidence of arterial thrombosis in patients with a Val at position 247 of β2GPI (which may interact with domain V (Yasuda *et al*, 2005)), the effect of polymorphic changes on the biochemistry of β2GPI needs to be expanded away from an examination of phospholipid binding.

In the cohort examined here, the association between the Cys306Gly polymorphism and arterial events was maintained in the aPL positive group but not in the aPL negative group. Other studies have shown that in Cys306Gly heterozygotes, the total (and therefore functional) binding of their β2GPI is

normal (Gushiken *et al*, 2002). Therefore, the aPL present in these patients should be able to transduce whatever effects are mediated via β2GPI binding normally. The presence of the polymorphic variant of β2GPI in these individuals may however, be exerting an additional pro-coagulant effect as discussed above.

Chapter 7 General Discussion

In this thesis, I have presented an analysis of the biochemistry of β2GPI with a particular focus on the proteolytic cleavage of the protein by plasmin (and other proteolytic enzymes) and looked at the effect of a common polymorphic variant of the protein in a large patient cohort. The work presented here has gone on to examine the interaction of antiphospholipid antibodies in relation to their effects on aspects of the fibrinolytic process.

In chapter 3, I have described part of the APS positive patient cohort at UCLH. This work has allowed me to "field test" a newly proposed serological classification for APS. A particular concern of mine with this new classification was the proposed removal of the anticardiolipin assay. In analysing our cohort, I demonstrated that a proportion of patients were positive for anticardiolipin activity alone and suggested that the assay should therefore be retained especially for clinical use. The data here does illustrate however that these "aCL only" patients do in the main tend to have low titre aPL and perhaps some caution needs to be exerted when they are diagnosed with "definite APS". This caution should particularly be aimed at insuring that it is established that these patients are persistently aPL positive. Since the work for this thesis was carried out, an international consensus statement has suggested another serological classification for APS (Miyakis *et al*, 2006). This classification is as follows: I One or more laboratory criteria present (aCL; anti-β2GPI; LA); IIa LA alone; IIb aCL alone; IIc anti-β2GPI alone.

In chapters 4 and 5, samples of IgG containing aPL activity from selected high titre patients from our cohort were used to investigate aspects of the interaction between aPL and components of the fibrinolytic system. In much of

the work presented in chapters 4 and 5, purified IgG fractions from either pooled normal plasma or patients with APS were compared in a range of fibrinolytic assays. I wanted to keep my experiments as simple as possible in terms of design and I felt that isolating the likely pathogenic component from the plasma was the best way to achieve this. By "spiking" plasma or purified reagents with IgG fractions, I was able to remove other variables from the experimental systems. This would not have been feasible if whole plasma or serum from patients was used.

In chapter 4, a novel method to quantify the reduction in negatively charged phospholipid binding of β2GPI cleaved by plasmin in domain V was developed. This assay was then used to test whether aPL can interfere with this process. Some of the patient samples examined showed activity of this nature but to a modest degree. However, this interference does illustrate the potential for aPL to protect their main cofactor protein from proteolytic damage. Variations in the structure of domain V of β2GPI were also demonstrated to be less susceptible to cleavage by plasmin. This finding adds another layer to the biochemistry of β2GPI polymorphic variants. Further work in these areas should involve a larger patient cohort than that used in this study, where only six IgG fractions were examined. In addition, an attempt should be made to quantify clipped β2GPI levels in those patients with samples showing a reduction in cleavage effect, to determine if this action is significantly active *in vivo*. It would also be interesting to examine this activity in an assay with a cellular surface present to determine whether the protective effect of the aPL is greater when a binding surface for β2GPI is present.

Data was also presented in chapter 4 illustrating that kallikrein and factor Xa can also reduce β2GPI binding to phospholipid and in the case of kallikrein that the resulting protein is of a similar molecular weight and antigenic structure to that produced by plasmin. The effect of kallikrein compared to plasmin was of a smaller magnitude. However, this action of kallikrein does open a potential mechanism of negative feedback on some of the pro-fibrinolytic effects of kallikrein via the inhibitory action of clipped β2GPI on plasminogen activation.

In chapter 5, the effect of aPL on the cellular binding of plasminogen was investigated. This work has shown that some aPL are able to reduce the binding of plasminogen to the surface of HUVEC. Interestingly since the experimental work for this thesis was completed, another group have demonstrated that a proportion of patients with APS have antibody activity directed against the tPA/plasminogen co-receptor annexin II (Cesarman-Maus *et al*, 2006). My work does not confirm that the effect seen in this thesis is due to an interaction of aPL with annexin II; one must remember that plasminogen can bind to other cell surface components. Bearing the work of this group in mind, however, I would suggest that anti-annexin II activity is sought in our patient samples showing an effect on plasminogen binding. Another antibody activity perhaps to look for would be that against S100A10, which forms part of the annexin II heterotetramer. Currently much work in APS research is focusing on annexin II both at the level of fibrinolytic component binding and relating to the interaction of the protein with β2GPI (Meroni *et al*, 2004). Other future work that should be carried out in this area would be to determine whether β2GPI is needed as a cofactor in the experimental system used in chapter 5.

Work was also presented in chapter 5 demonstrating a reduction in fibrinolysis in a urokinase activated plasma clot lysis system in the presence of aPL. Previous studies have demonstrated an effect of aPL in such systems. However, to the best of my knowledge, this is the first example of such an effect in a uPA activated sample. The magnitude of the effect seen here was again small, and further work should aim to examine this effect in the presence of higher IgG concentrations. This perhaps should be an aim for all of the purified IgG experiments presented in this thesis.

The work in chapter 6 did not demonstrate an association between the presence of the Cys306Gly polymorphism of β2GPI and the production (or lack of) aPL. Unexpectedly, the data produced on subgroup analysis suggested an association between the polymorphism and arterial pathology. Unfortunately because the study was not initially set up to determine an arterial risk factor, I cannot present data to prove that this polymorphism is an independent risk factor for arterial events. The data presented here would indicate that a further prospective study should take place to investigate this further.

In conclusion, this work would support the hypothesis that part of the pathophysiology of APS involves an impairment of fibrinolytic processes. This however, does not seem to be a universal feature in all patients and may be of a small magnitude. This work has also further explored the physiology of proteolytic processing of β2GPI and illustrated that contact activation may be important in this process.

The pathophysiology of APS at present seems complex and to consist of multiple mechanisms. Perhaps we are observing a disease, that by virtue of

leading to the production of antibodies which can bind to different cell types and different parts of the cell surface, does indeed have multiple pathogenic mechanisms at play. This may be the reason for the myriad of clinical associations with the condition. Moreover, new levels of the physiology of the main cofactor for aPL, β2GPI, are emergent and perhaps as more of these are revealed, our understanding of this condition will increase.

Publications arising from this thesis

- **Peer reviewed papers**

MJ Nash, RS Camilleri, S Kunka, IJ Mackie, SJ Machin and H Cohen
The anticardiolipin assay is required for sensitive screening for antiphospholipid antibodies.
Journal of Thrombosis and Haemostasis 2:1077-1081 (2004).

MJ Nash, RS Camilleri, R Liesner, IJ Mackie, SJ Machin, H Cohen
Paradoxical association between the 316 Trp to Ser Beta2 Glycoprotein I polymorphism and anti-Beta2 Glycoprotein I antibodies.
British Journal of Haematology 2003;120:529-531

- **Abstract Publications**

Oral Presentations

XI International congress on Antiphospholipid Antibodies, Sydney 2004
MJ Nash, IJ Mackie and SJ Machin.
Kallikrein is able to specifically cleave Beta2-glycoprotein I in domain V

XI International congress on Antiphospholipid Antibodies, Sydney 2004
MJ Nash, IJ Mackie, H Cohen and SJ Machin
Antiphospholipid antibodies can reduce the in vitro cleavage of domain V of Beta2-glycoprotein I by plasmin.

European Antiphospholipid Forum – London 2004
MJ Nash, RS Camilleri, T Everington, D Peebles and H Cohen
Pregnancy outcome of women with antiphospholipid antibodies associated with late obstetric complications treated with aspirin and low molecular weight heparin

European Antiphospholipid Forum – London 2004
MJ Nash, RS Camilleri, S Kunka, IJ Mackie, SJ Machin and H Cohen.
The role of the anticardiolipin assay in the classification of the antiphospholipid syndrome.

Scientific and Standardisation Subcommittee, 49th Congress of the International Society on Thrombosis and Haemostasis
MJ Nash, RS Camilleri, S Kunka, IJ Mackie, SJ Machin and H Cohen
Analysis of >100 patients with persistent aPL according to new serological criteria

Poster Presentations

44th meeting of The British Society for Haematology (2004):
An analysis of the beta2glycoprotein I Cys306Gly polymorphism in patients with thrombotic and obstetric disorders.
MJ Nash, RS Camilleri, IJ Mackie, SJ Machin and H Cohen.

44th meeting of The British Society for Haematology (2004):
An analysis of the antiphospholipid antibody status of 111 women with late pregnancy morbidity.
MJ Nash, RS Camilleri, T Everington and H Cohen.

49th Congress of the International Society on Thrombosis and Haemostasis (2003)
MJ Nash, RS Camilleri, S Kunka, IJ Mackie, SJ Machin, H Cohen.
A proposed ISTH revised classification for the Antiphospholipid Syndrome

49th Congress of the International Society on Thrombosis and Haemostasis (2003)
MJ Nash, R Camilleri, R Leisner, I Mackie, SJ Machin, H Cohen.
Paradoxical association between the 316 Trp to Ser beta 2-glycoprotein I polymorphism and anti-beta2GPI antibodies.

43rd meeting of The British Society for Haematology (2003):
MJ Nash, RS Camilleri, S Kunka, IJ Mackie, SJ Machin, H Cohen.
The role of anticardiolipin antibodies (aCL) in the diagnosis of the antiphospholipid syndrome.

10th International Congress on Antiphospholipid Antibodies (2002).
R Camilleri, MJ Nash, R Leisner, I Mackie, SJ Machin, H Cohen.
TRP316SER Beta2-glycoprotein I variant with reduced binding to phospholipid associated with anti-beta-2-glycoprotein I antibodies in a patient with pregnancy complications.

List of manufacturers used in this thesis

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